Doc Code - SEQ.XML

WIPO Type Code -

SLIC Placeholder Sheet for P-ELP (CMS) Content

Application Number: 18361782 Document Date: 07/28/2023

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- Sequence Listing

At the time of document entry (noted above):

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- External customers may access CMS content via PAIR/Patent Center Retrieval using the Supplemental Content tab.

Form Revision Date: 07 January 2022

DocCode - SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 18361782 Document Date: 07/28/2023

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- Drawing

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- USPTO employees may access SCORE content via DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

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ELECTRONIC ACKNOWLEDGEMENT RECEIPT

APPLICATION #	RECEIPT DATE / TIME	ATTORNEY DOCKET #
18/361,782	**07/28/2023 09:15:59 PM ET**	**SRZN-006/04US 328202-2203**

Title of Invention

WNT SURROGATE MOLECULES AND USES THEREOF

Application Information

APPLICATION TYPE	Utility - Nonprovisional Application under 35 USC 111(a)	PATENT #	-
CONFIRMATION #	9001	FILED BY	Donna Doyle
PATENT CENTER #	62527099	FILING DATE	-
CUSTOMER #	157070	FIRST NAMED INVENTOR	Yang LI
CORRESPONDENCE ADDRESS	-	AUTHORIZED BY	Carol Laherty

Documents

TOTAL DOCUMENTS: 8

DOCUMENT	PAGES	DESCRIPTION	SIZE (KB)
SRZN-006_04US-Application_Data_Sheet.pdf	12	Application Data Sheet	2174 KB
SRZN-006_04US-Power_of_Attorney.pdf	2	Power of Attorney	998 KB
SRZN-006_04US-Drawings_1-59.pdf	59	Drawings-only black and white line drawings	16326 KB
SRZN-006_04US-Drawings_60-132.pdf	73	Drawings-only black and white line drawings	11542 KB
SRZN_006_04US_ST26.xml	-	Sequence Listing (ST.26 XML File)	2899 KB

SRZN-006_04US - CON application.pdf		207	-	1116 KB
SRZN-006_04US - CON application-SPEC.pdf	(1-195)	195	Specification	1171 KB
SRZN-006_04US - CON application-CLM.pdf	(196-206)	11	Claims	122 KB
SRZN-006_04US - CON application-ABST.pdf	(207-207)	1	Abstract	74 KB

Digest

DOCUMENT	MESSAGE DIGEST(SHA-512)
SRZN-006_04US-Application_Data_Sheet.pdf	25F7C996857107038CFE8F8A380BAD480DB001563BE6E88195E61EBF801F32A05A3C408218F10E8EAE56A7B4FD01CC324834C1D433FF1A8014DBDAF127B2C758
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SRZN-006_04US-Drawings_1-59.pdf	02CB31AB1AAAA50B663EBF930790F2AF922F953382BAC6A99FA22BC9E21FF5875CA2E3A55238557BB1CF572C546C4D327805556FD55E3E945B35CFF743D1CFF7
SRZN-006_04US-Drawings_60-132.pdf	A8CDAA33E87DF0ACFC00EEF512CFED9657C4F21472B5DB1CD80CE2EC92EC1E4BC5D2FA8907065EF8B4C26600A338418460FD28205A629F5190059CF677230E1D
SRZN_006_04US_ST26.xml	63A3F79A61748E9E852A9201086B25B515162F56C8AC53920CCA0C36D23F08CA2E04295218B80D5C7182BFF056A00BCA5078A6D3557AF2827A613A592AB8B1ED
SRZN-006_04US - CON application.pdf	7F8BDE9CCD2C35EEFA94015D87A8608F874286772B22E8B52EBC7C9309B80D12BD05A5006001982A14CEE9596F898412C3

	C24A04BAF2D38D49E8DF605E4CFBB2
SRZN-006_04US - CON application-SPEC.pdf	4D8C7D7C38F62D052D379D5064B5C4B0B6E64AAD5E54B12C 0C6B2ECEC7282B0AEE9FCE171BF3682288A0B431CE6A881F3 2AA78426A28CACB209742657CB3ECF6
SRZN-006_04US - CON application-CLM.pdf	0C49E5A9F38481CC2C802EFA9C139A6E7C5DAE21899730EE7 8385A9DFF8100386578FBF38F295985269B36B44F059365B56A 932EDBFB5F766E02B7AE46F4DC8C
SRZN-006_04US - CON application-ABST.pdf	0A983CE2F7DC116EE10A0DDFA515D4DA5AFF6B9DD54524B4 A5AB1D6BF712CE16422F916ACE7FCBB3BFBB8A4CE2497B8C 6873B6EF87F41B0EB13FDB4259D47B44

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

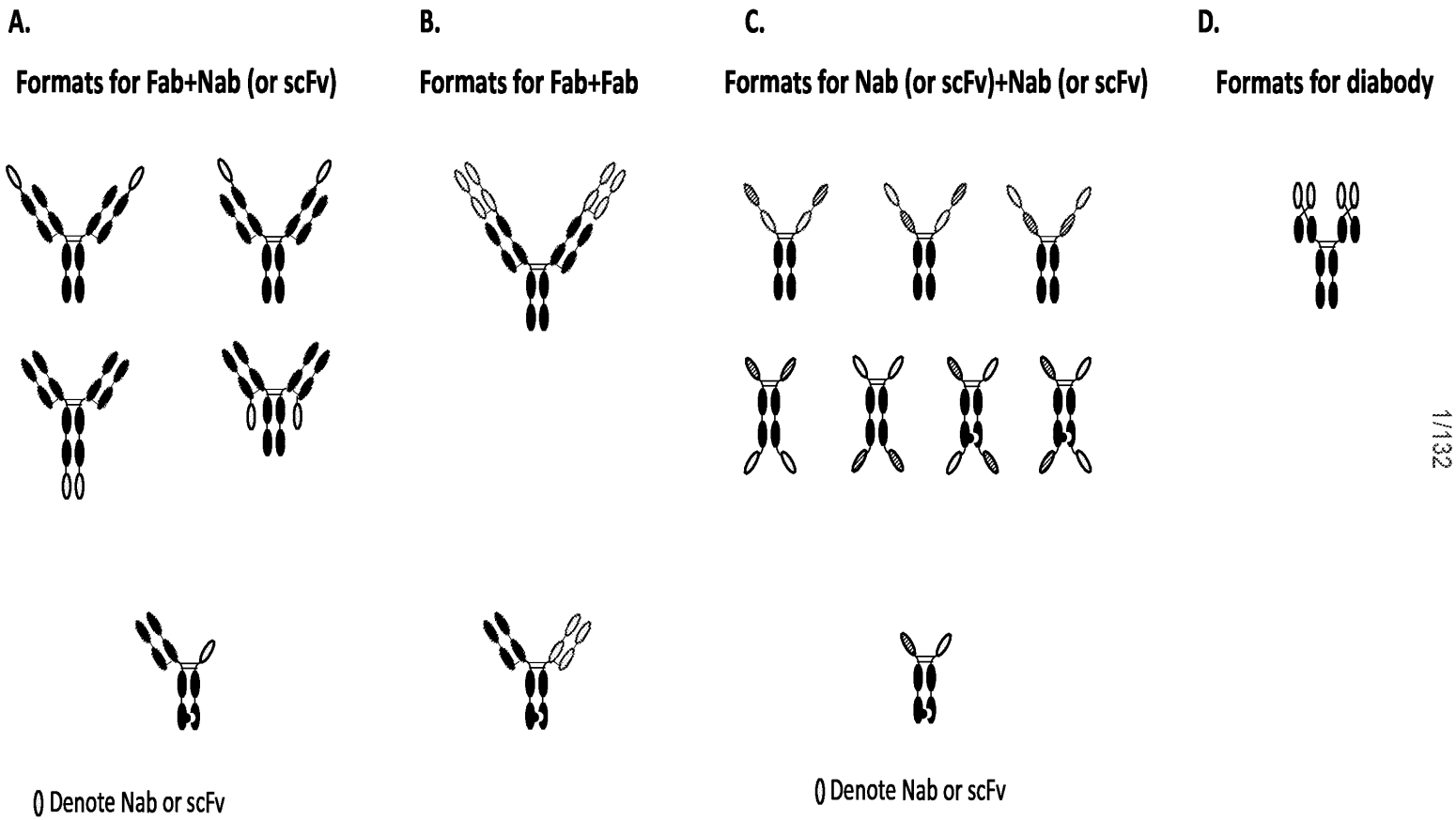


FIG. 1

MS&D Exhibit 1008, Page 6 of 4223

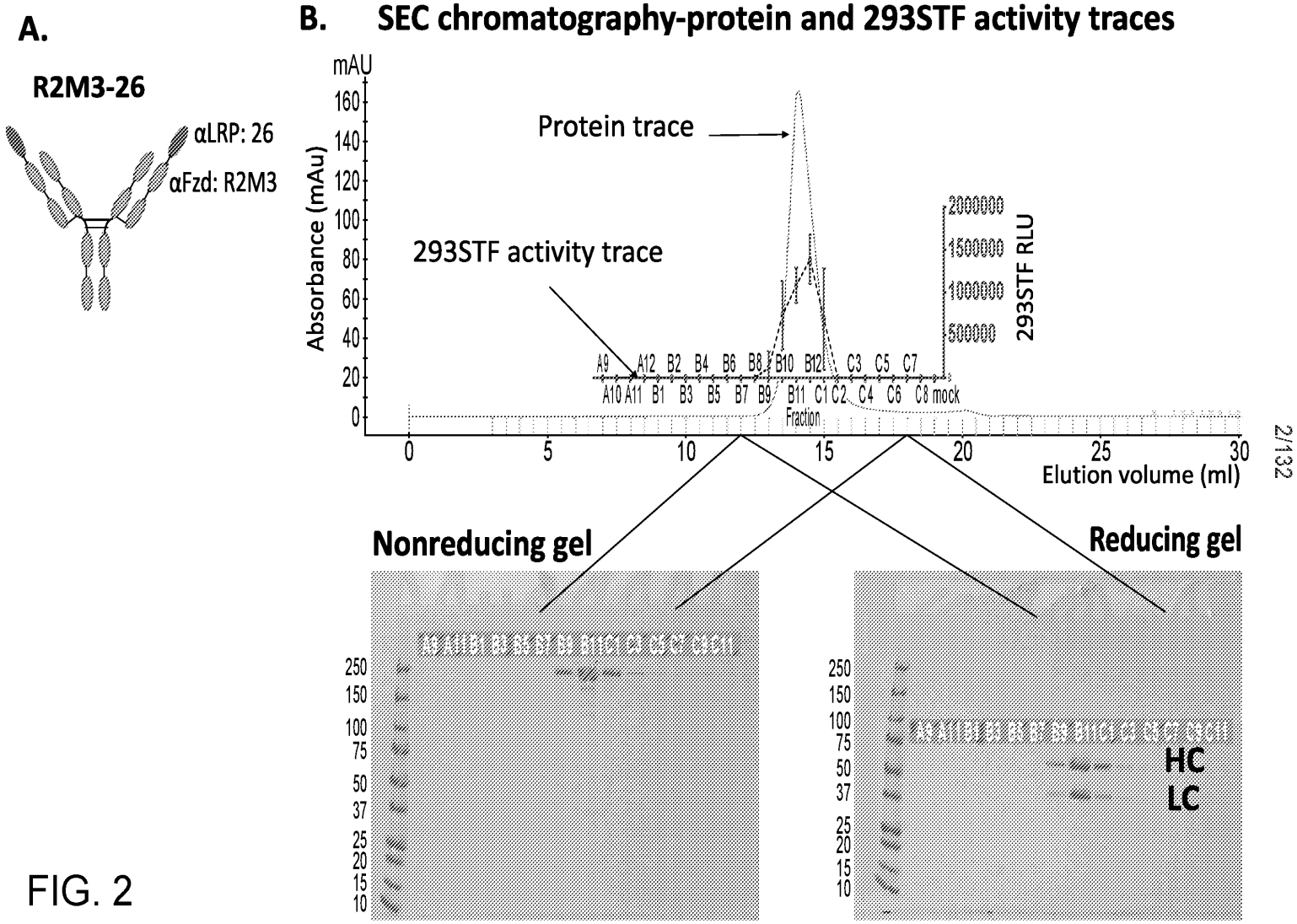


FIG. 2

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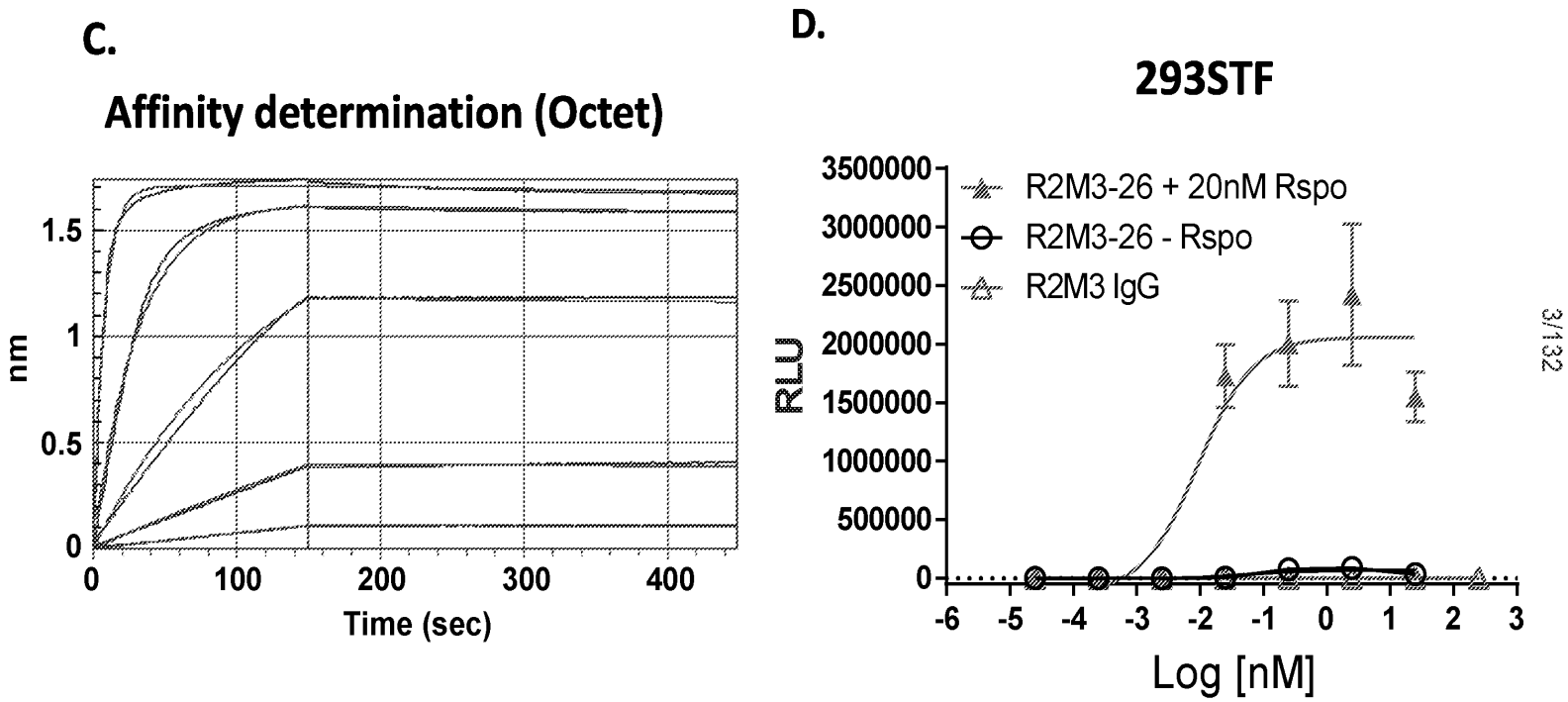


FIG. 2 (contd.)

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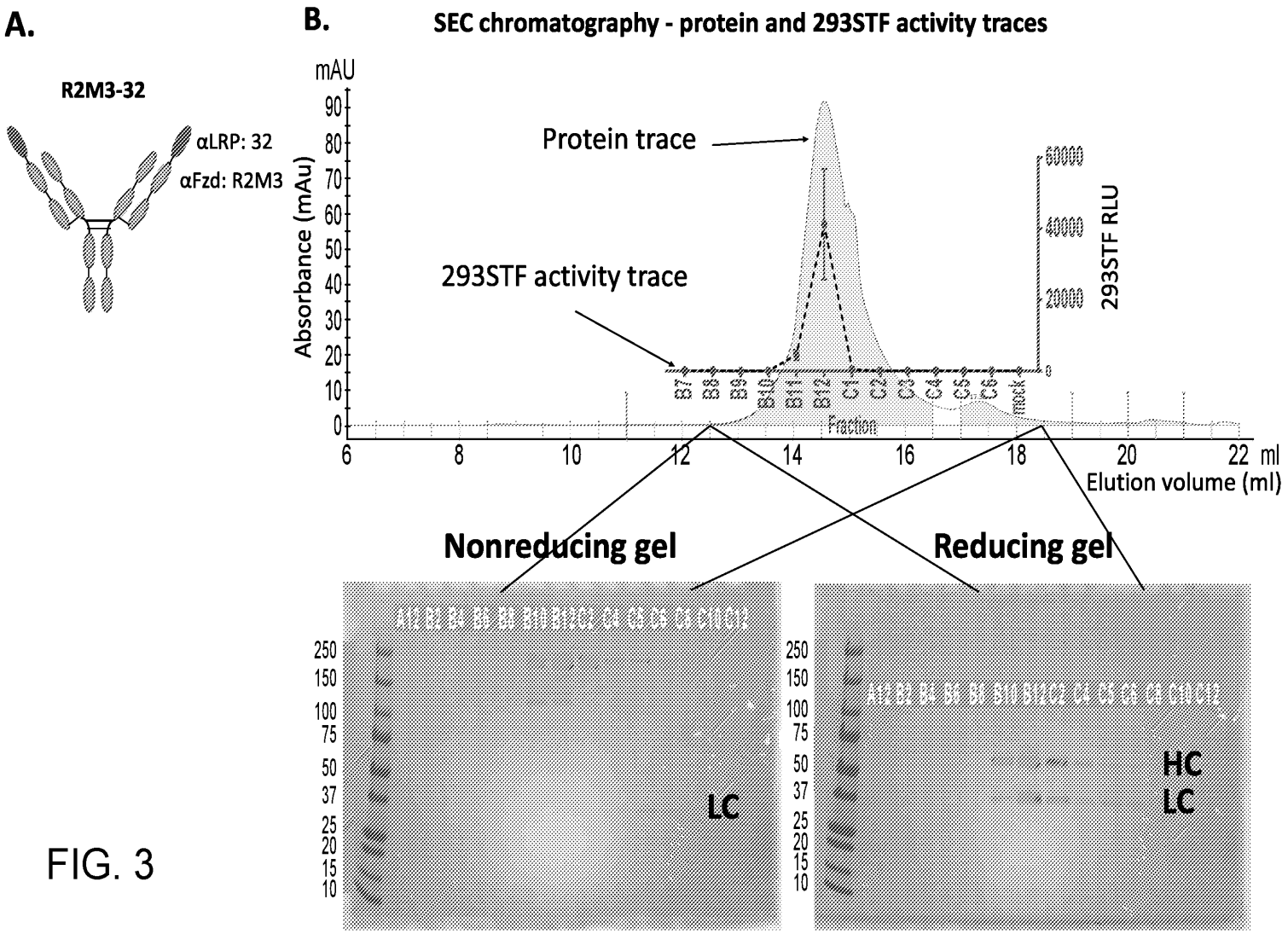

A.
R2M3-32
αLRP: 32
αFzd: R2M3
B.
SEC chromatography - protein and 293STF activity traces
mAU
Absorbance (mAu)
Protein trace
293STF activity trace
293STF RLU
Fraction
Elution volume (ml)
Nonreducing gel
Reducing gel
LC
HC
LC

FIG. 3

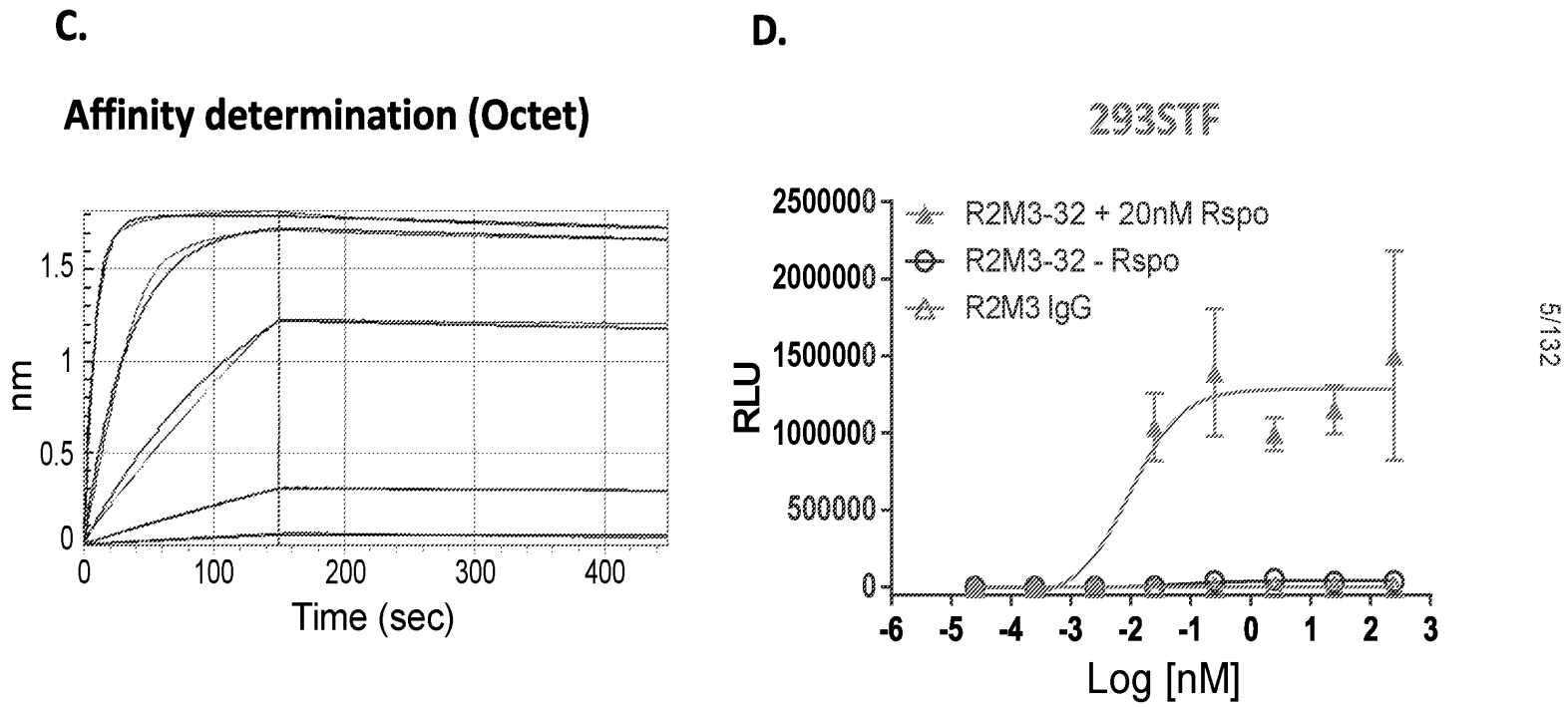
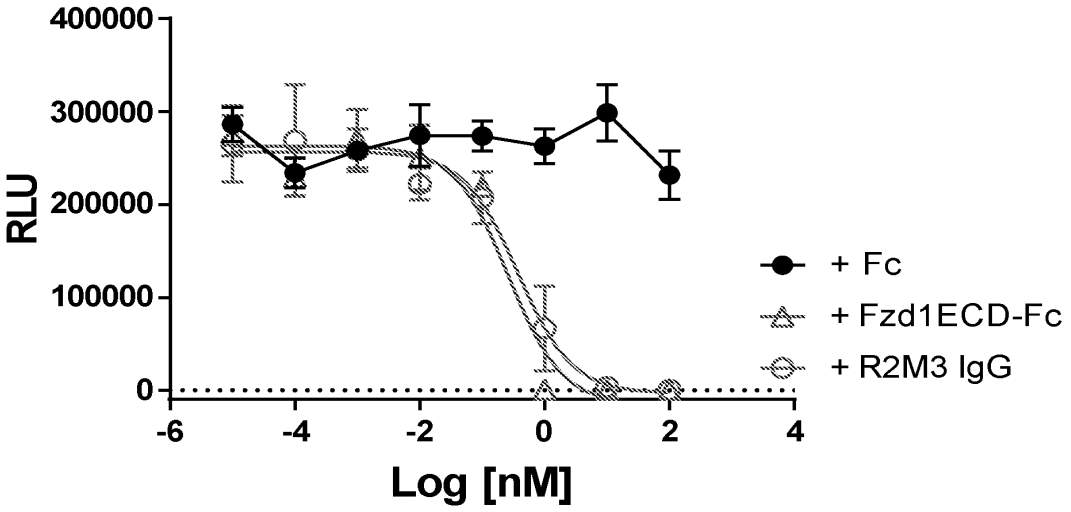


FIG. 3 (contd.)

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A.


400000
300000
200000
100000
0
RLU
-6
-4
-2
0
2
4
Log [nM]
+ Fc
+ Fzd1ECD-Fc
+ R2M3 IgG

B.

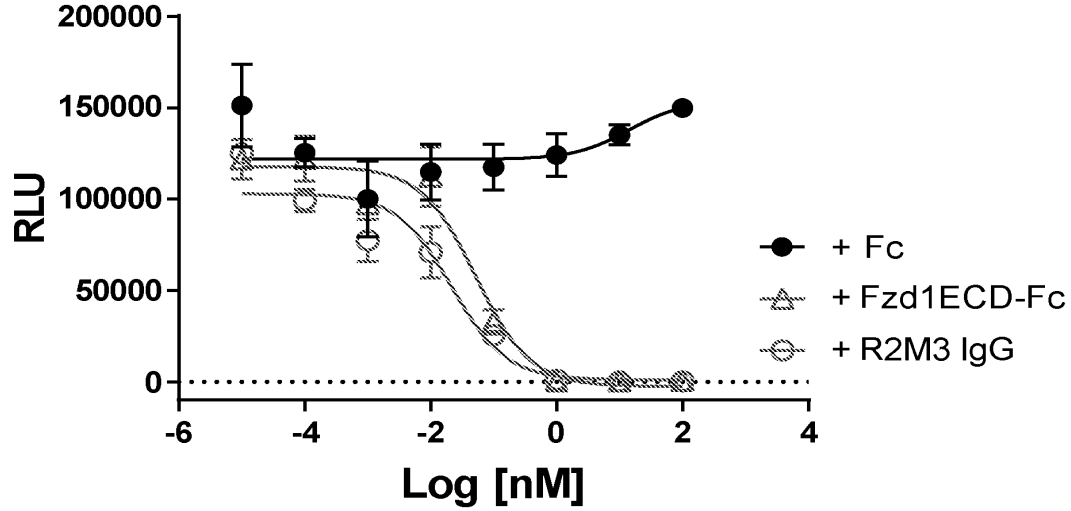

200000
150000
100000
50000
0
RLU
-6
-4
-2
0
2
4
Log [nM]
+ Fc
+ Fzd1ECD-Fc
+ R2M3 IgG

FIG. 4

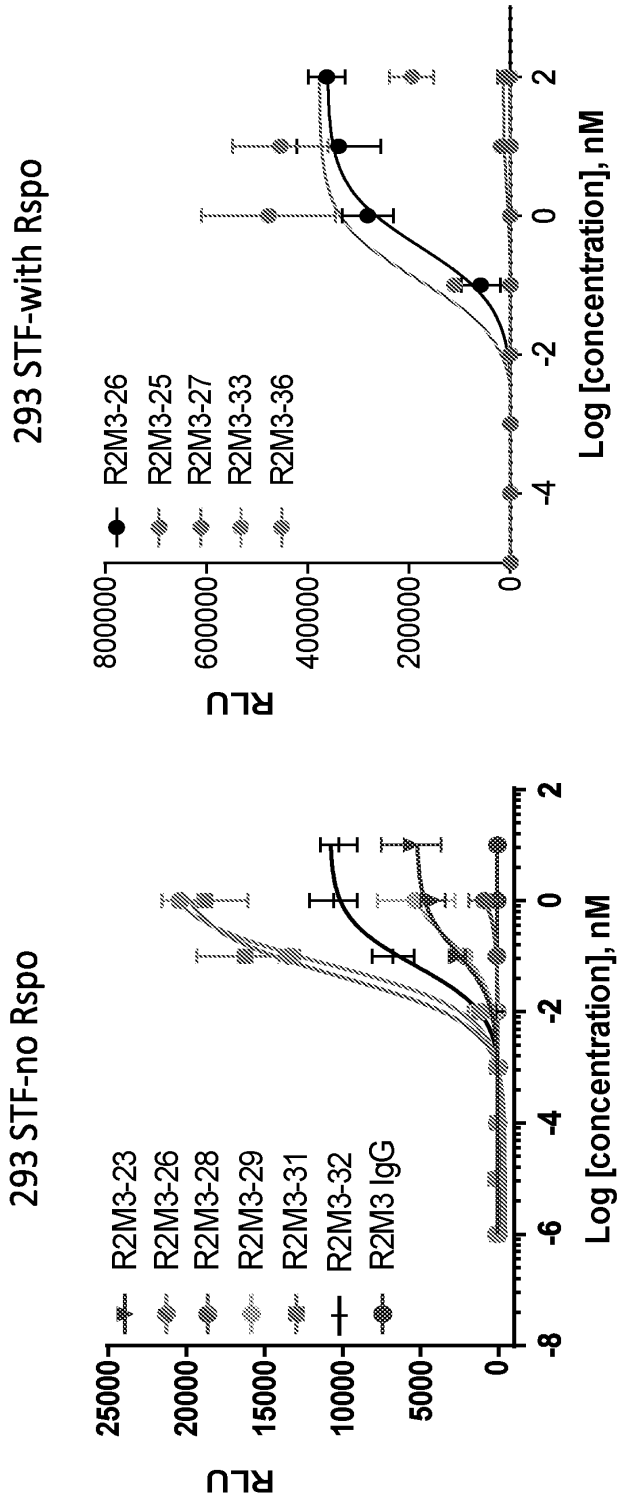

293 STF-no Rspo
RLU
25000
20000
15000
10000
5000
0
-8
-6
-4
-2
0
2
Log [concentration], nM
R2M3-23
R2M3-26
R2M3-28
R2M3-29
R2M3-31
R2M3-32
R2M3 IgG
293 STF-with Rspo
RLU
800000
600000
400000
200000
0
-4
-2
0
2
Log [concentration], nM
R2M3-26
R2M3-25
R2M3-27
R2M3-33
R2M3-36

FIG. 5

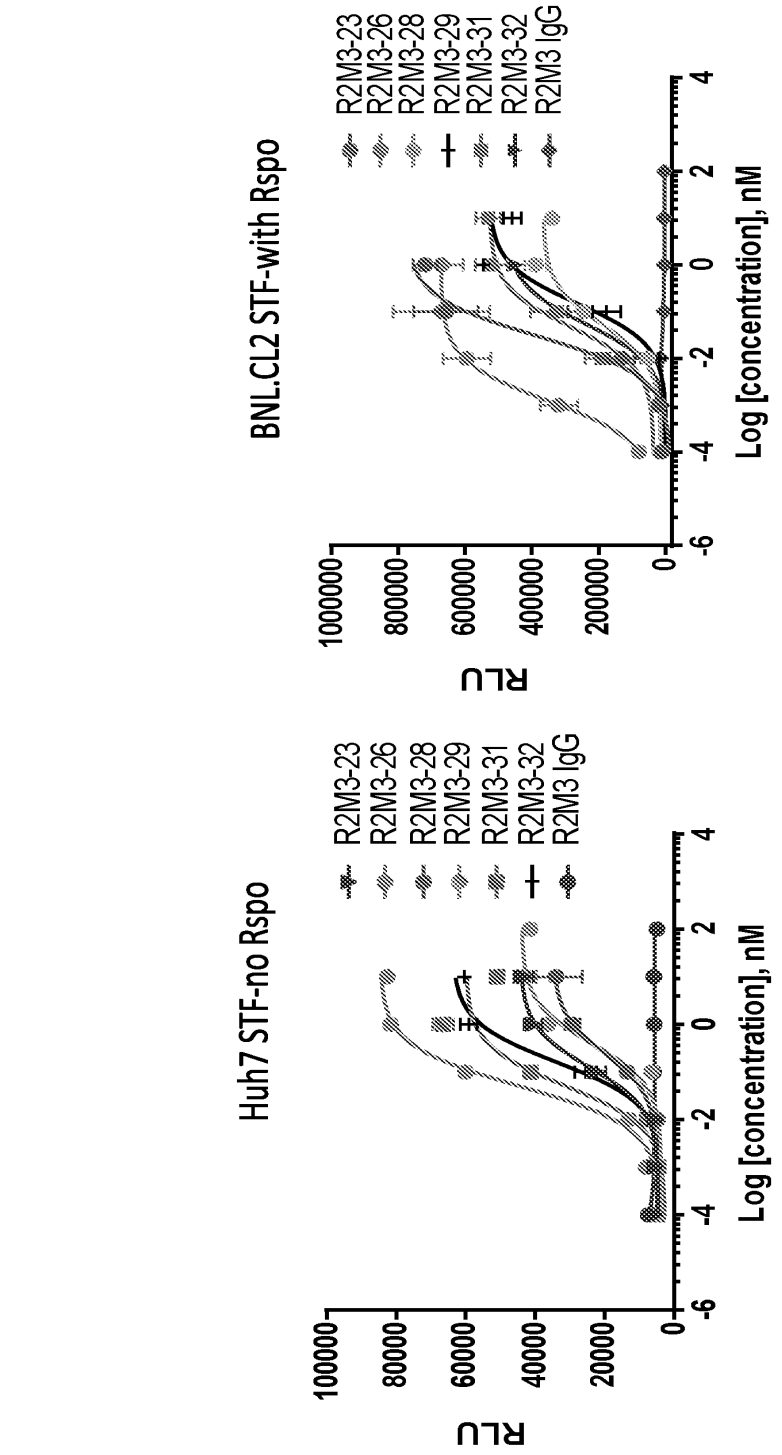

Huh7 STF-no Rspo
BNL.CL2 STF-with Rspo
RLU
Log [concentration], nM
R2M3-23
R2M3-26
R2M3-28
R2M3-29
R2M3-31
R2M3-32
R2M3 IgG

FIG. 5 (contd.)

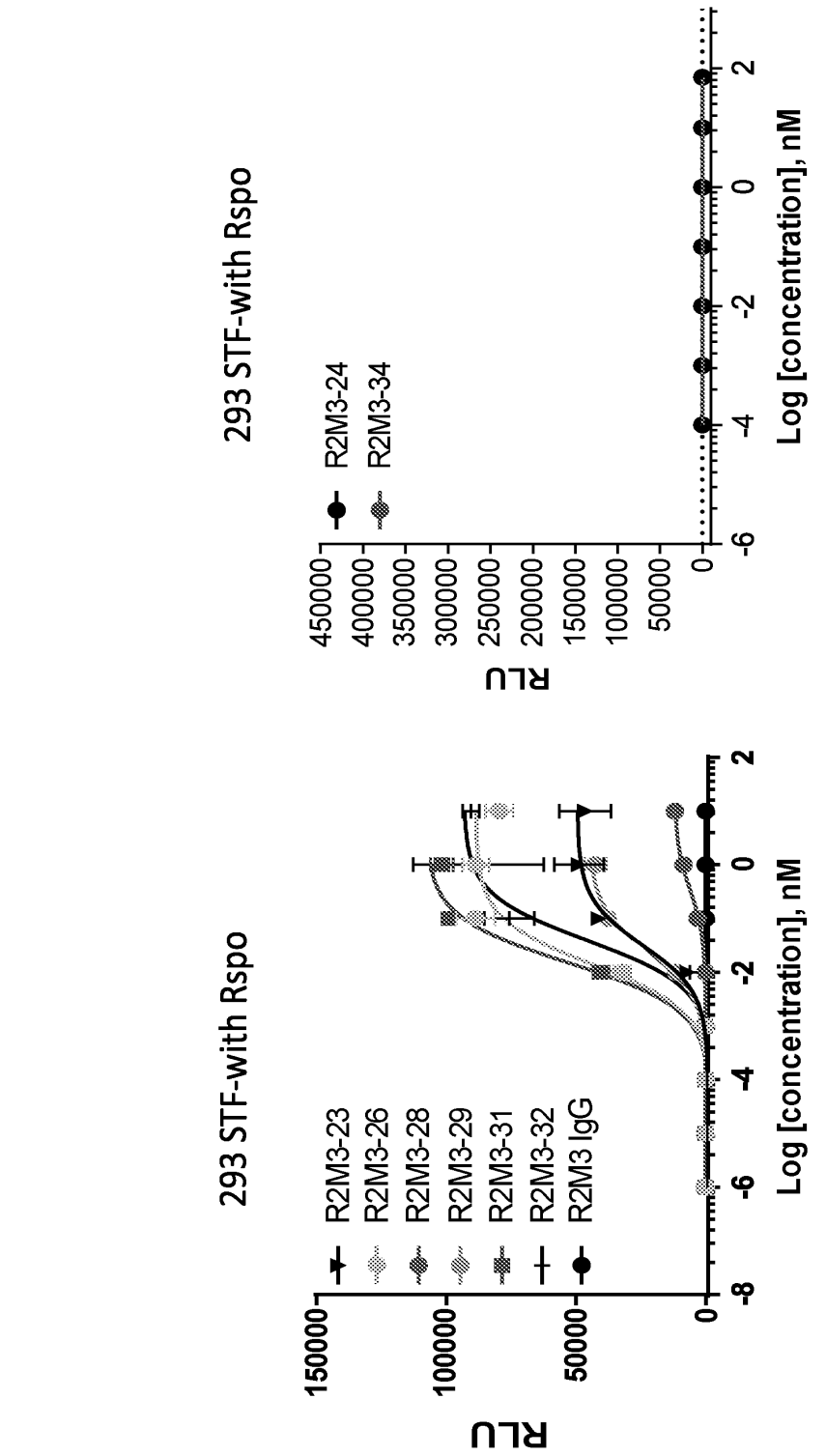

293 STF-with Rspo
R2M3-23
R2M3-26
R2M3-28
R2M3-29
R2M3-31
R2M3-32
R2M3 IgG
RLU
150000
100000
50000
0
-8
-6
-4
-2
0
2
Log [concentration], nM
293 STF-with Rspo
R2M3-24
R2M3-34
RLU
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
-6
-4
-2
0
2
Log [concentration], nM

FIG. 5 (contd.)

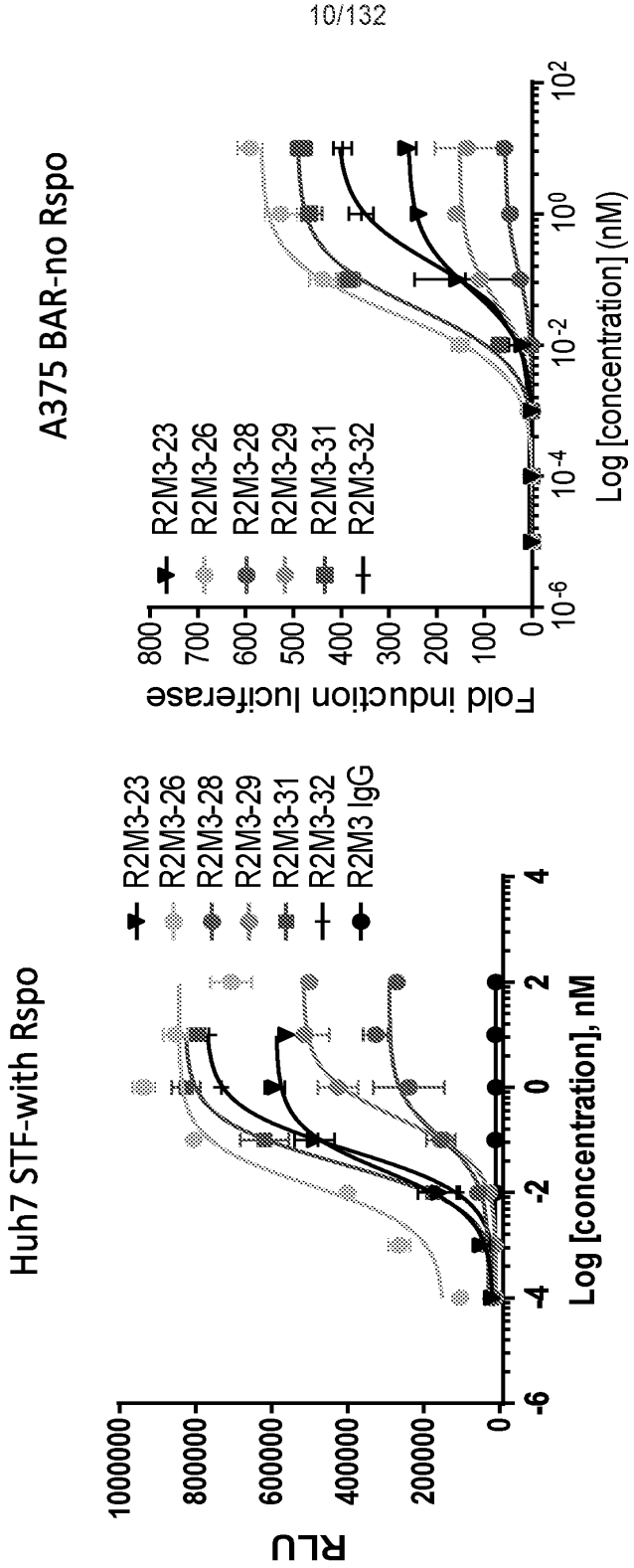

A375 BAR-no Rspo
Fold induction luciferase
800
700
600
500
400
300
200
100
0
R2M3-23
R2M3-26
R2M3-28
R2M3-29
R2M3-31
R2M3-32
10^{-6}
10^{-4}
10^{-2}
10^{0}
10^{2}
Log [concentration] (nM)
Huh7 STF-with Rspo
RLU
1000000
800000
600000
400000
200000
0
R2M3-23
R2M3-26
R2M3-28
R2M3-29
R2M3-31
R2M3-32
R2M3 IgG
-6
-4
-2
0
2
4
Log [concentration], nM

FIG. 5 (contd.)

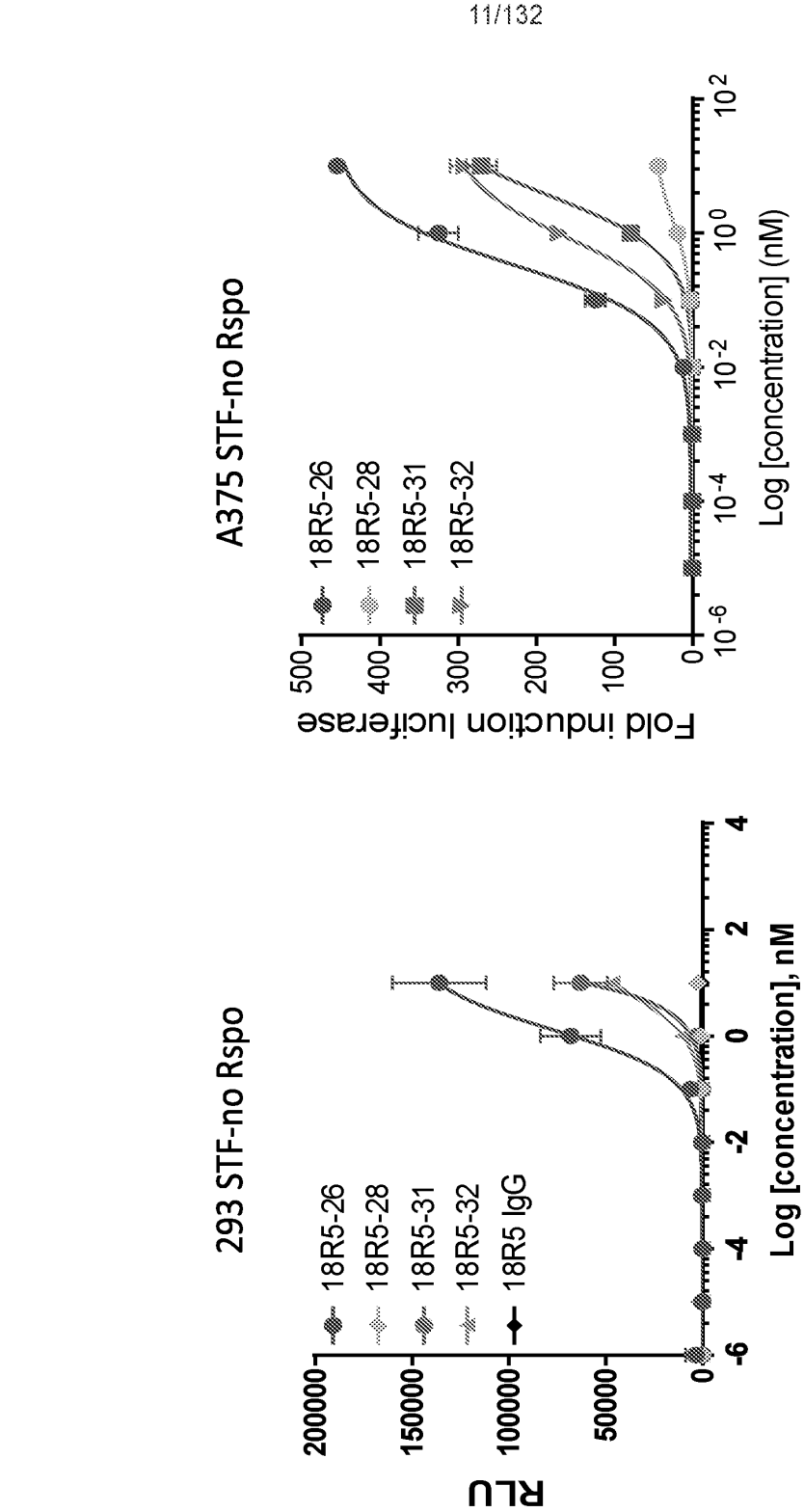

293 STF-no Rspo
RLU
200000
150000
100000
50000
0
18R5-26
18R5-28
18R5-31
18R5-32
18R5 IgG
-6
-4
-2
0
2
4
Log [concentration], nM
A375 STF-no Rspo
Fold induction luciferase
500
400
300
200
100
0
18R5-26
18R5-28
18R5-31
18R5-32
10-6
10-4
10-2
100
102
Log [concentration] (nM)

FIG. 6

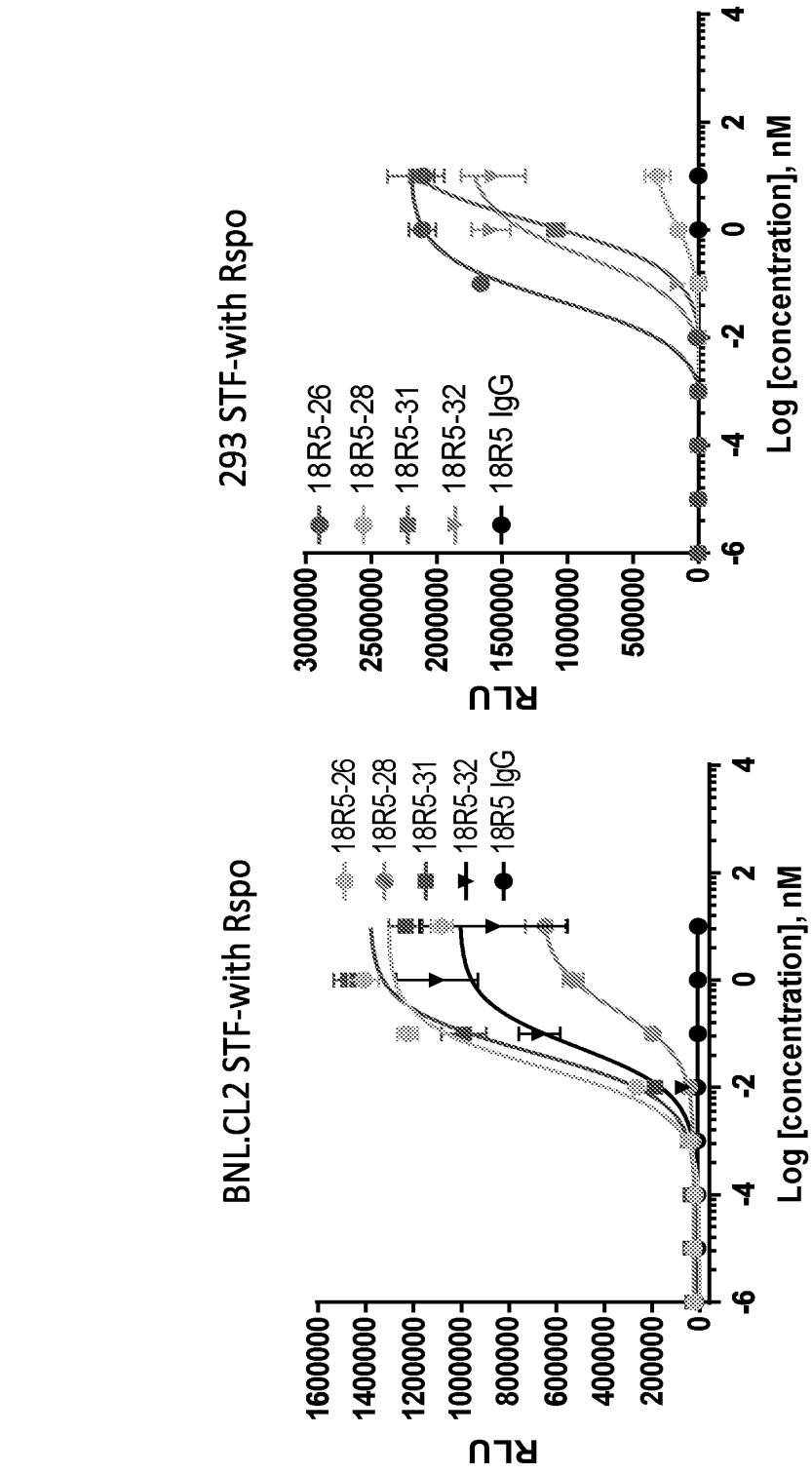

293 STF-with Rspo
RLU
3000000
2500000
2000000
1500000
1000000
500000
0
-6
-4
-2
0
2
4
Log [concentration], nM
18R5-26
18R5-28
18R5-31
18R5-32
18R5 IgG
BNL.CL2 STF-with Rspo
RLU
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
-6
-4
-2
0
2
4
Log [concentration], nM
18R5-26
18R5-28
18R5-31
18R5-32
18R5 IgG

FIG. 6 (contd.)

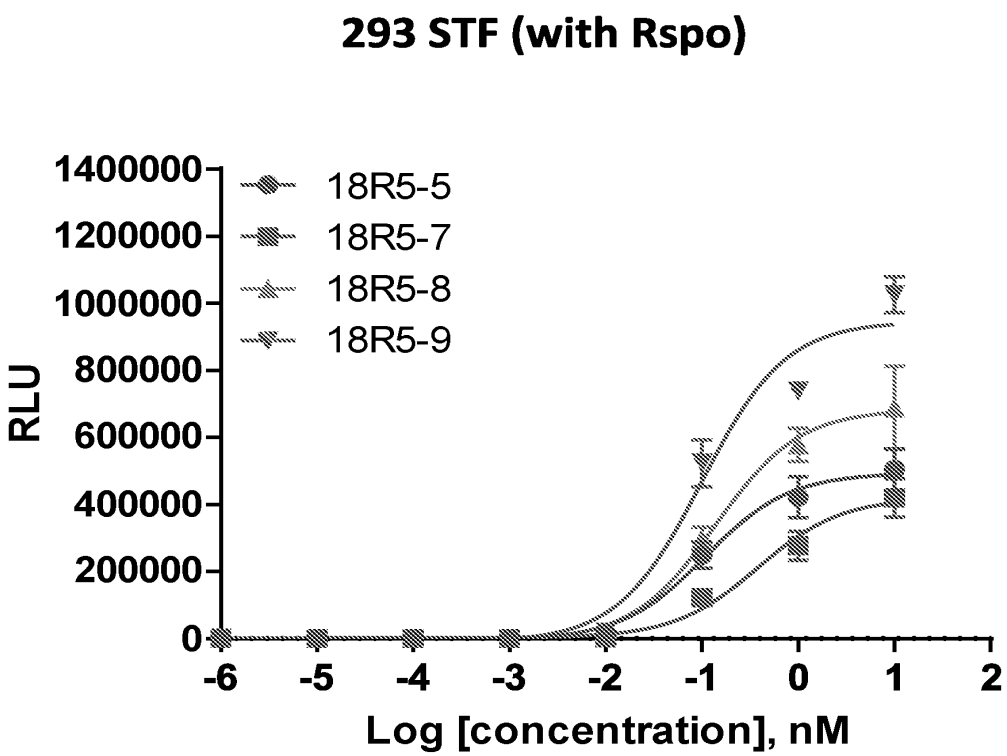

293 STF (with Rspo)
1400000
1200000
1000000
800000
600000
400000
200000
0
RLU
18R5-5
18R5-7
18R5-8
18R5-9
-6
-5
-4
-3
-2
-1
0
1
2
Log [concentration], nM

FIG. 7

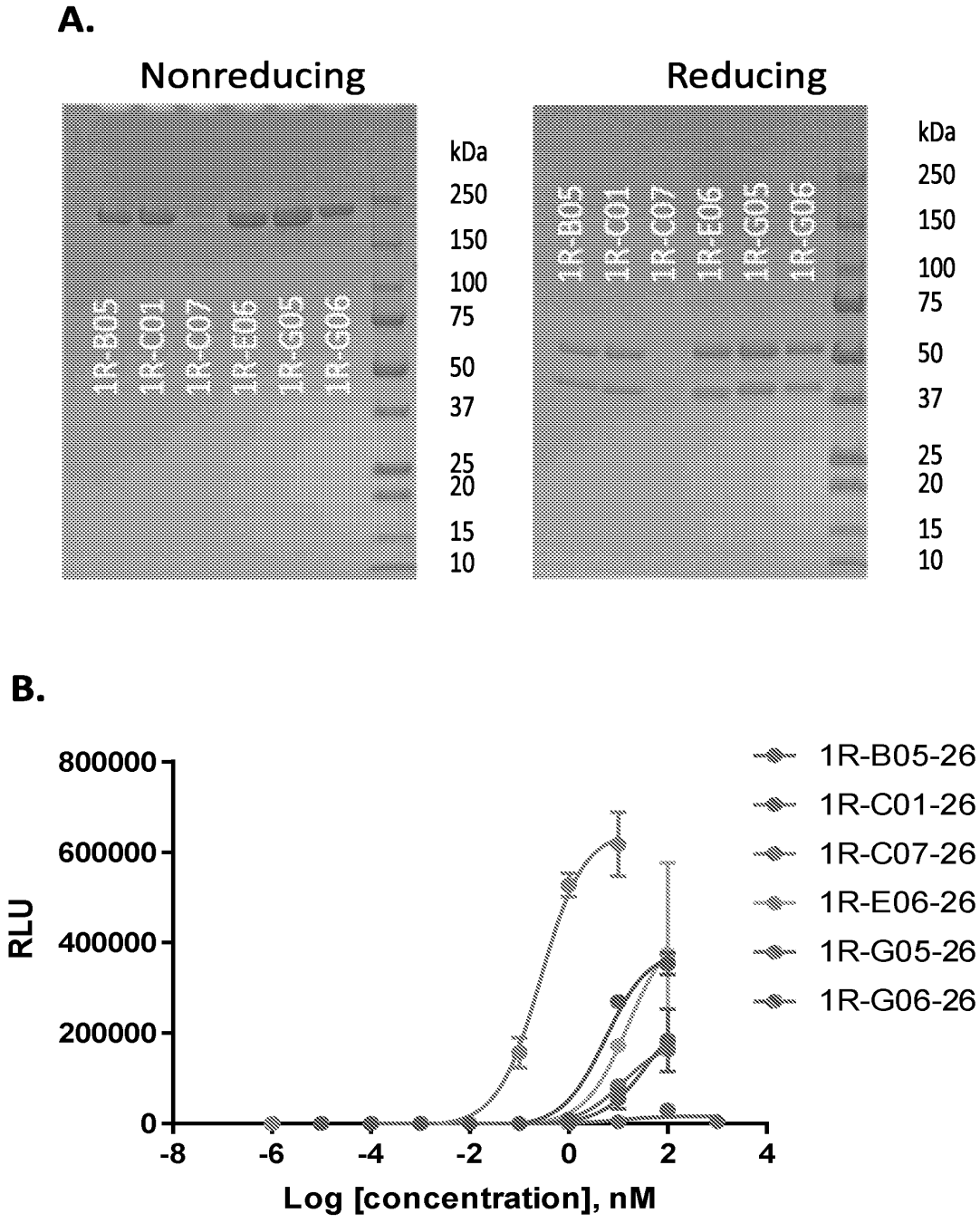
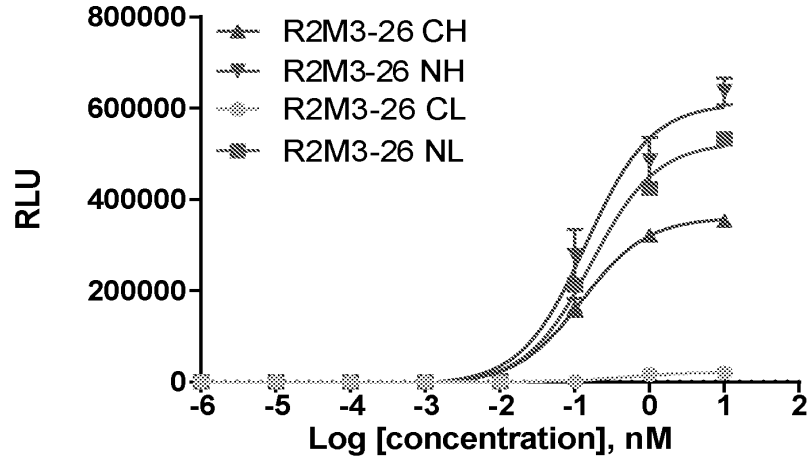
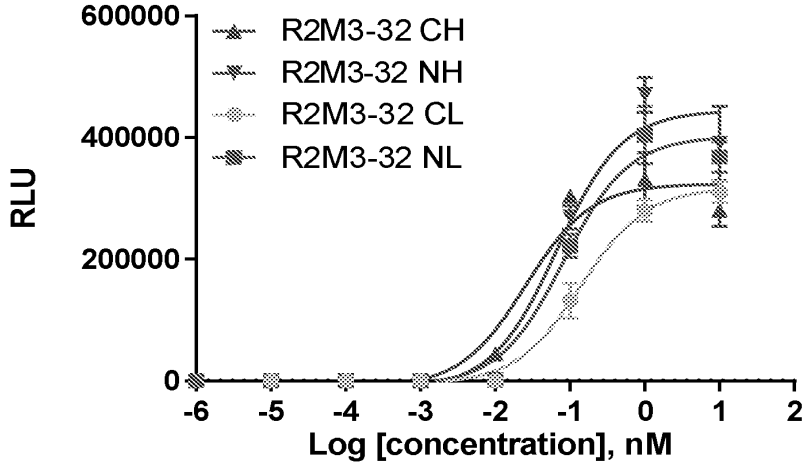

A.
Nonreducing
Reducing
kDa
250
150
100
75
50
37
25
20
15
10
1R-B05
1R-C01
1R-C07
1R-E06
1R-G05
1R-G06
B.
RLU
800000
600000
400000
200000
0
-8
-6
-4
-2
0
2
4
Log [concentration], nM
1R-B05-26
1R-C01-26
1R-C07-26
1R-E06-26
1R-G05-26
1R-G06-26

FIG. 8


800000
600000
400000
200000
0
RLU
R2M3-26 CH
R2M3-26 NH
R2M3-26 CL
R2M3-26 NL
-6
-5
-4
-3
-2
-1
0
1
2
Log [concentration], nM


600000
400000
200000
0
RLU
R2M3-32 CH
R2M3-32 NH
R2M3-32 CL
R2M3-32 NL
-6
-5
-4
-3
-2
-1
0
1
2
Log [concentration], nM

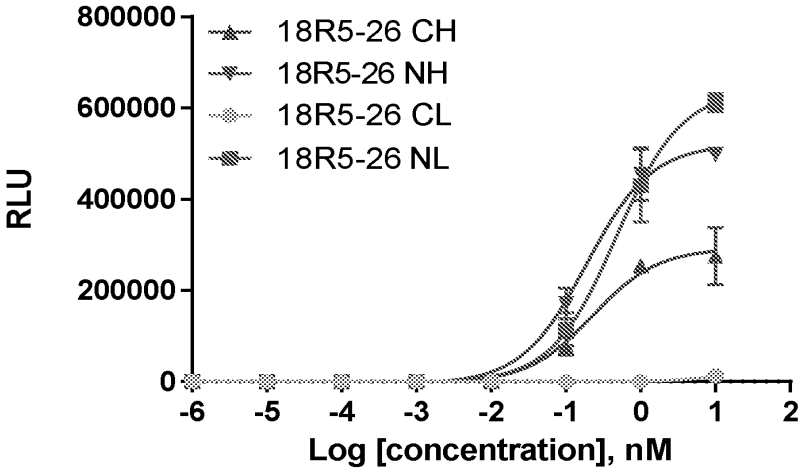

800000
600000
400000
200000
0
RLU
18R5-26 CH
18R5-26 NH
18R5-26 CL
18R5-26 NL
-6
-5
-4
-3
-2
-1
0
1
2
Log [concentration], nM

FIG. 9

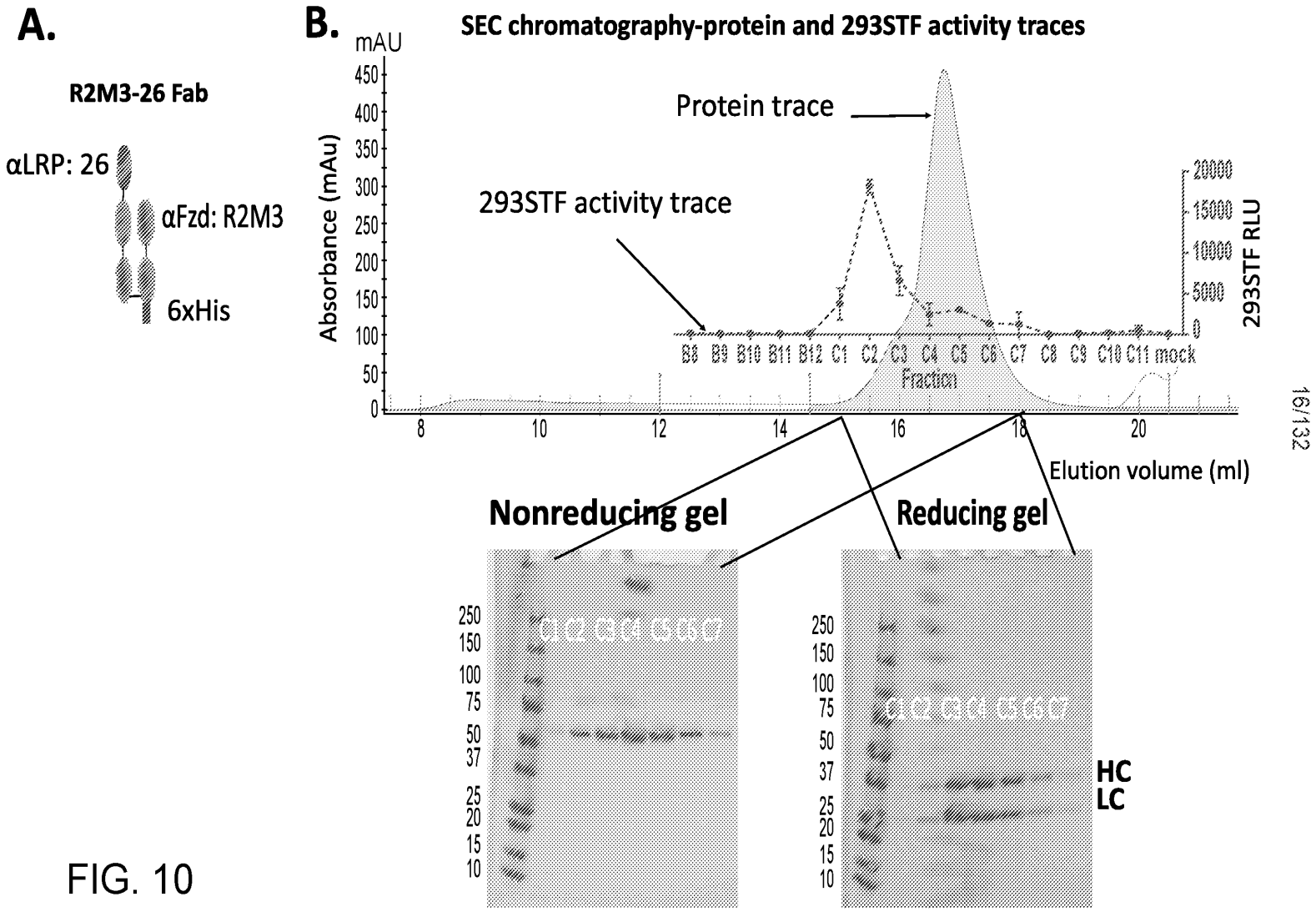

A.
R2M3-26 Fab
αLRP: 26
αFzd: R2M3
6xHis
B.
SEC chromatography-protein and 293STF activity traces
mAU
Absorbance (mAu)
Protein trace
293STF activity trace
293STF RLU
B8 B9 B10 B11 B12 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 mock
Fraction
Elution volume (ml)
Nonreducing gel
Reducing gel
HC
LC

FIG. 10

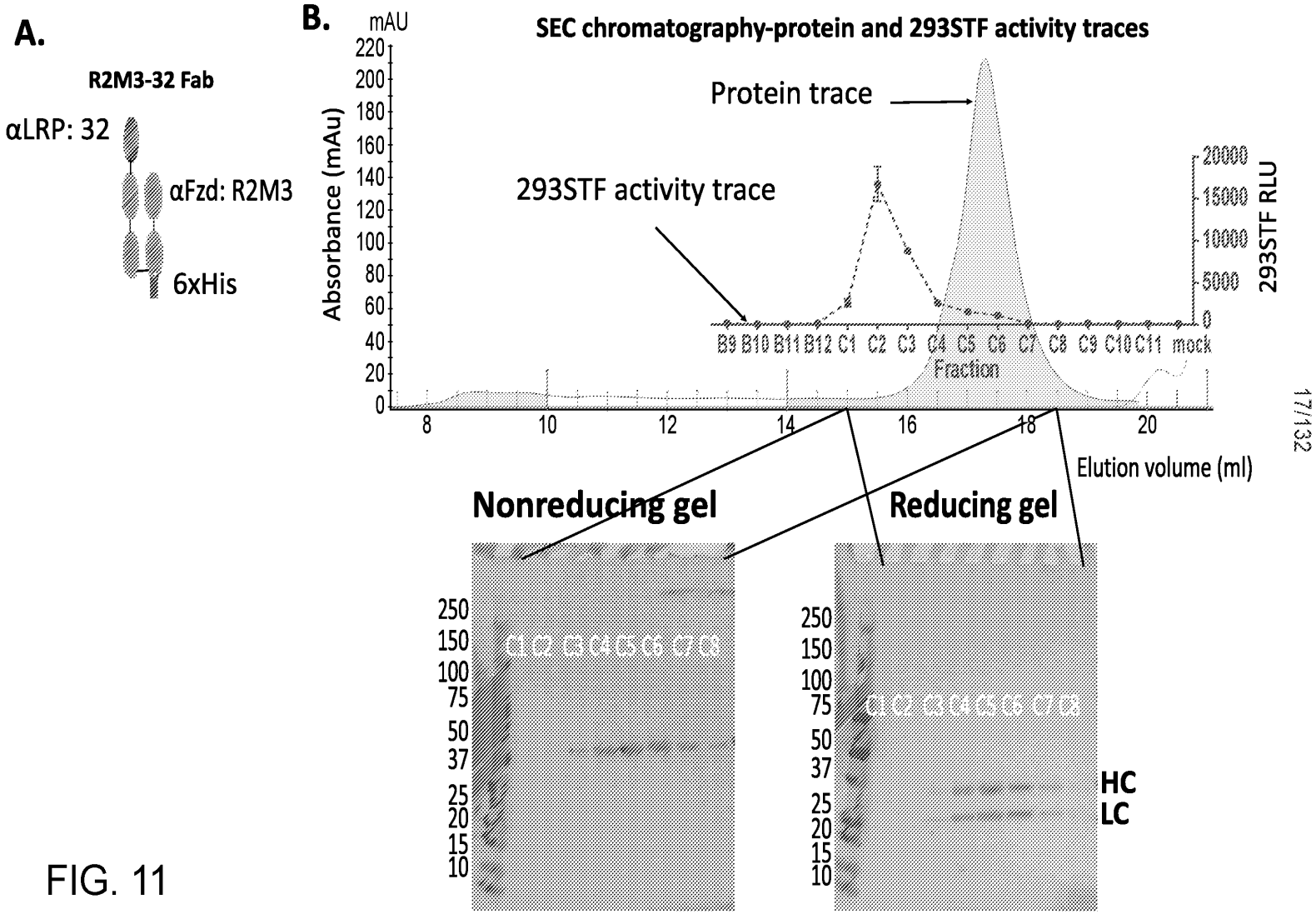

A.
R2M3-32 Fab
αLRP: 32
αFzd: R2M3
6xHis
B.
SEC chromatography-protein and 293STF activity traces
mAU
Absorbance (mAu)
Protein trace
293STF activity trace
293STF RLU
B9 B10 B11 B12 C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 mock
Fraction
Elution volume (ml)
Nonreducing gel
Reducing gel
HC
LC

FIG. 11

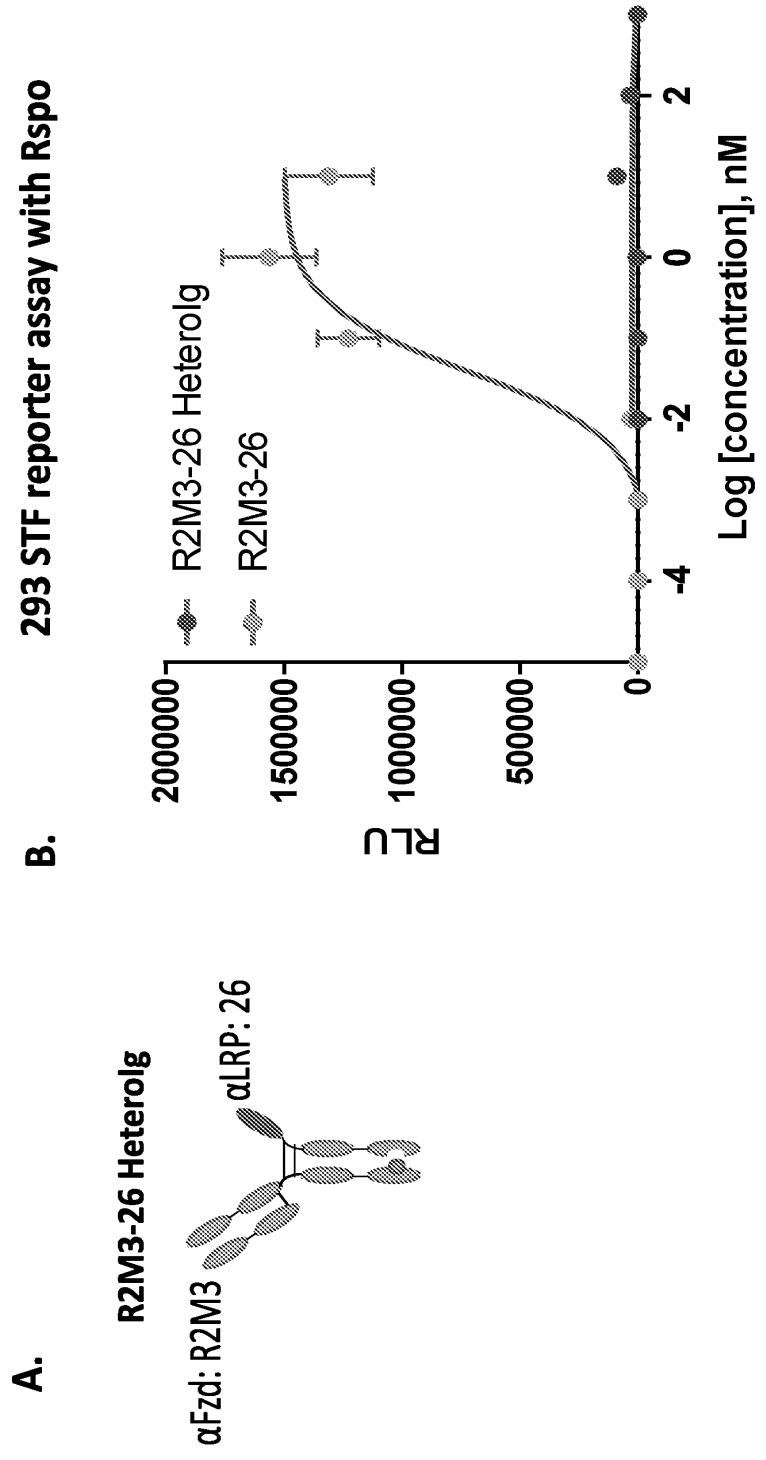

A.
R2M3-26 HeteroIg
αFzd: R2M3
αLRP: 26
B.
293 STF reporter assay with Rspo
R2M3-26 HeteroIg
R2M3-26
RLU
2000000
1500000
1000000
500000
0
-4
-2
0
2
Log [concentration], nM

FIG. 12

A.

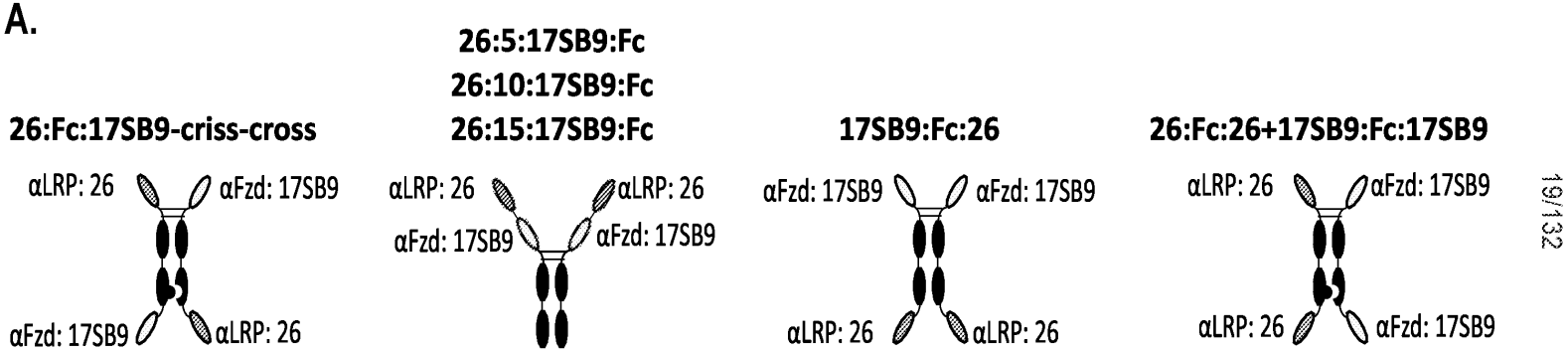


FIG. 13

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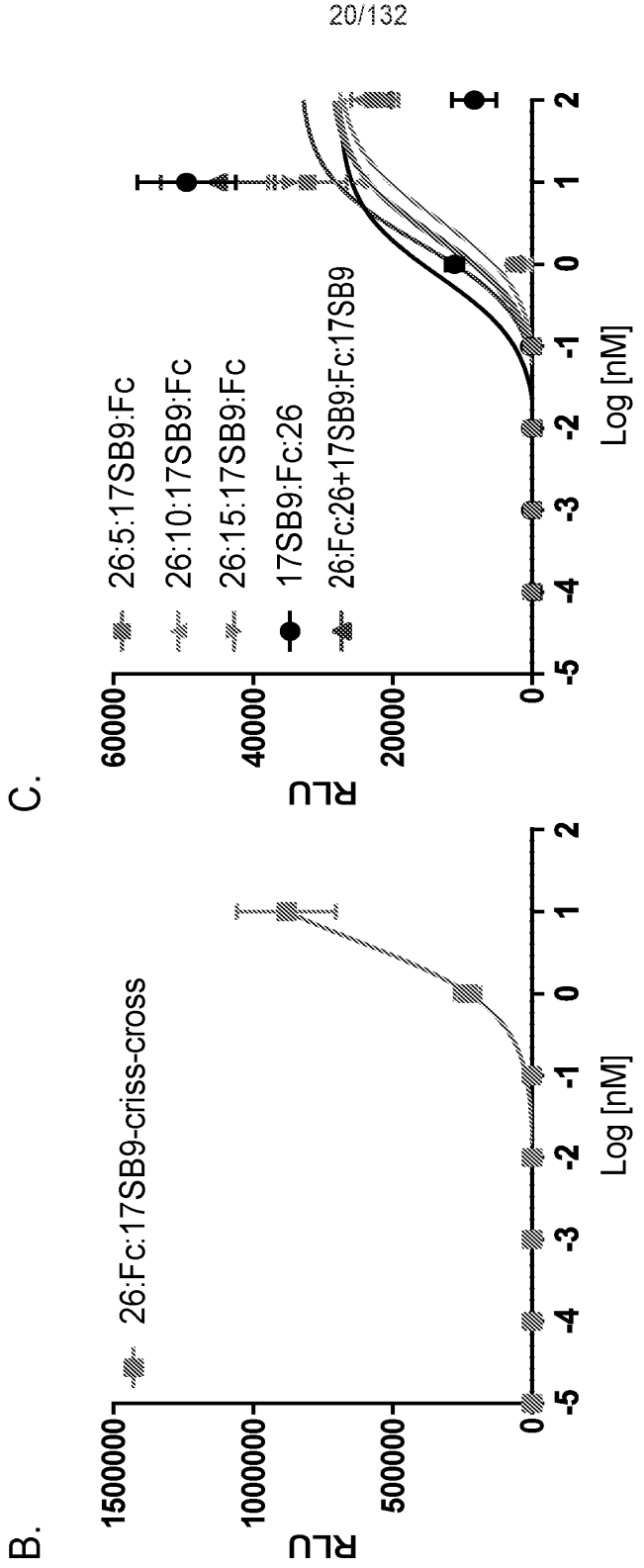
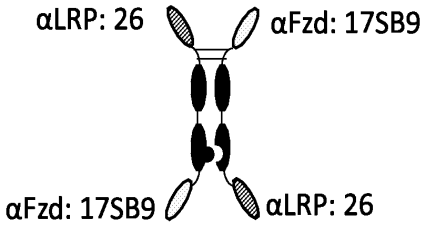

B.
C.
RLU
Log [nM]
26:Fc:17SB9-criss-cross
26:5:17SB9:Fc
26:10:17SB9:Fc
26:15:17SB9:Fc
17SB9:Fc:26
26:Fc:26+17SB9:Fc:17SB9

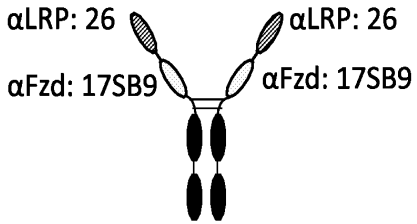
FIG. 13 (contd.)

D.

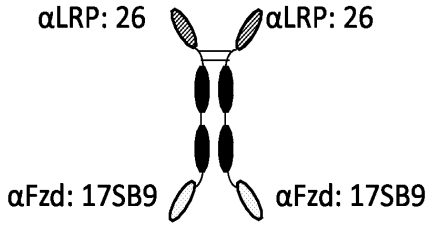
26:Fc:17SB9-criss-cross



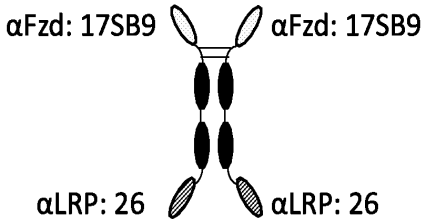
26:5:17SB9:Fc
26:10:17SB9:Fc
26:15:17SB9:Fc



26:Fc:17SB9



17SB9:Fc:26



26:Fc:26+17SB9:Fc:17SB9

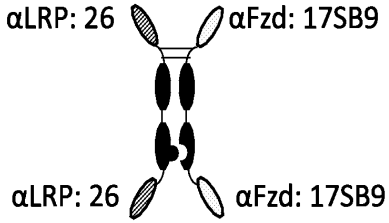


FIG. 13 (contd.)

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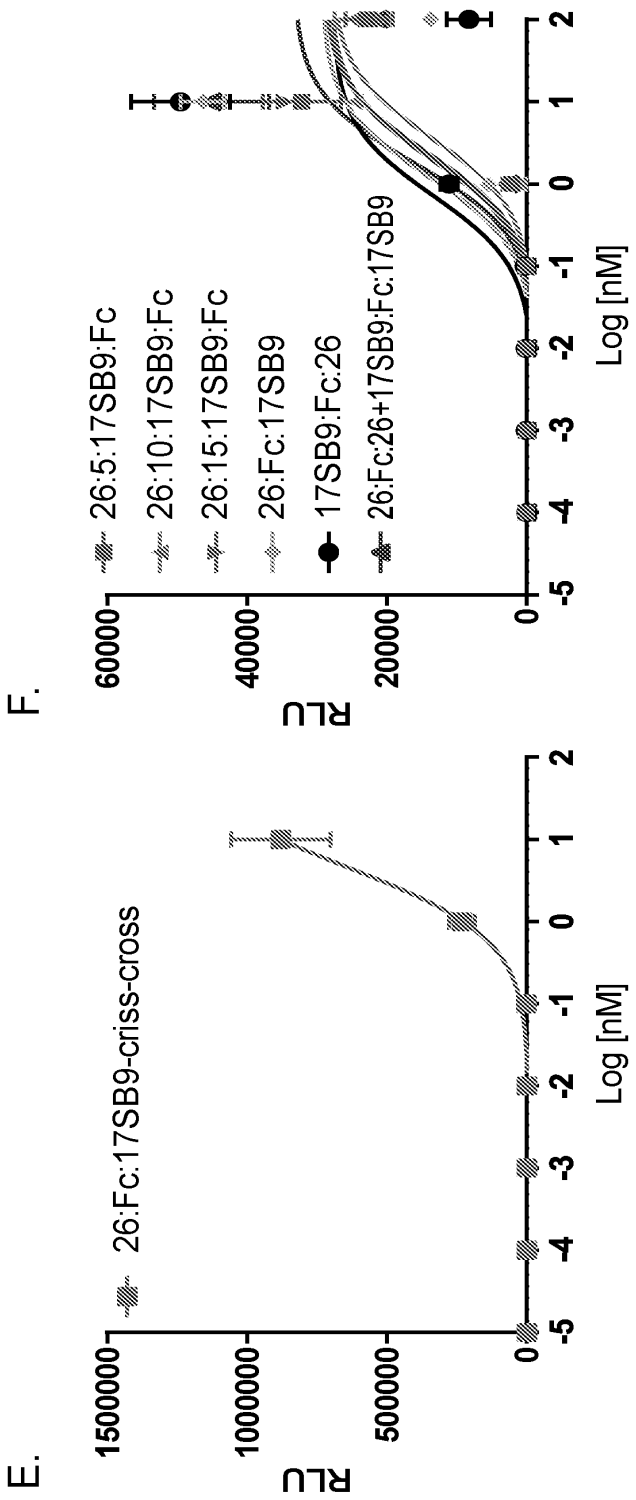

E.
F.
RLU
Log [nM]
26:Fc:17SB9-criss-cross
26:5:17SB9:Fc
26:10:17SB9:Fc
26:15:17SB9:Fc
26:Fc:17SB9
17SB9:Fc:26
26:Fc:26+17SB9:Fc:17SB9

FIG. 13 (contd.)

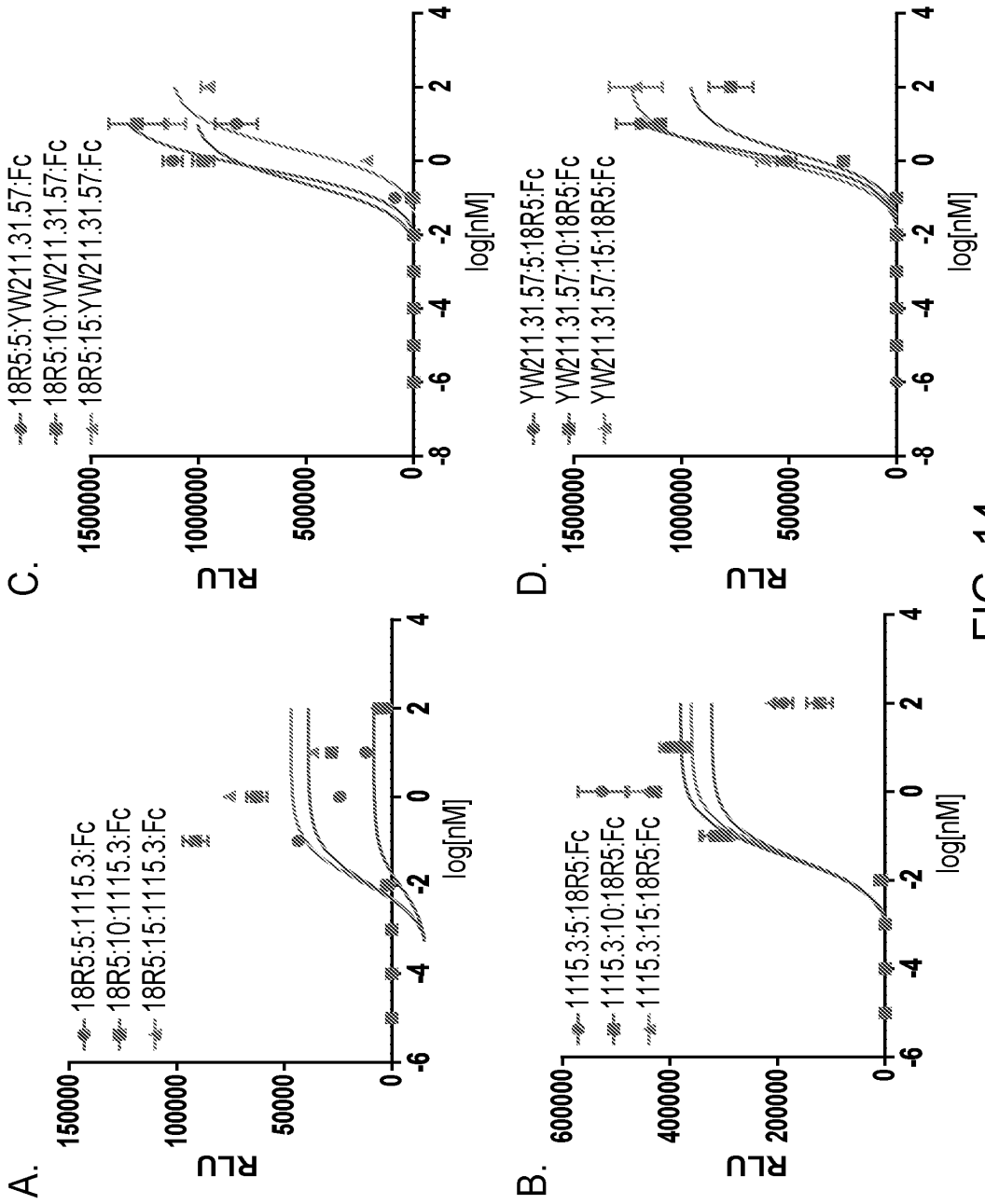
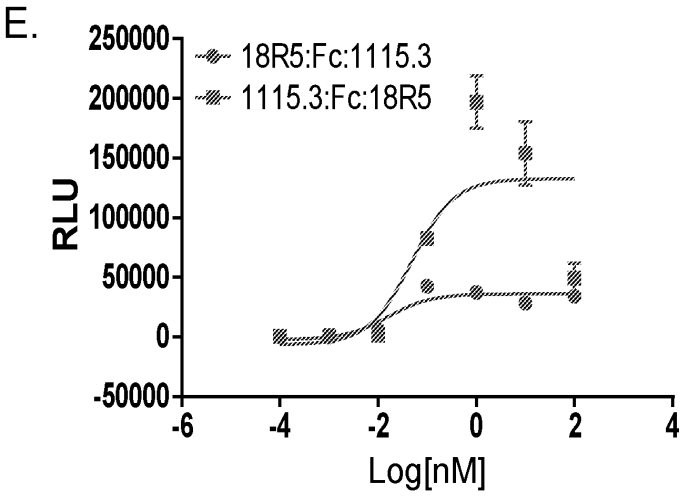
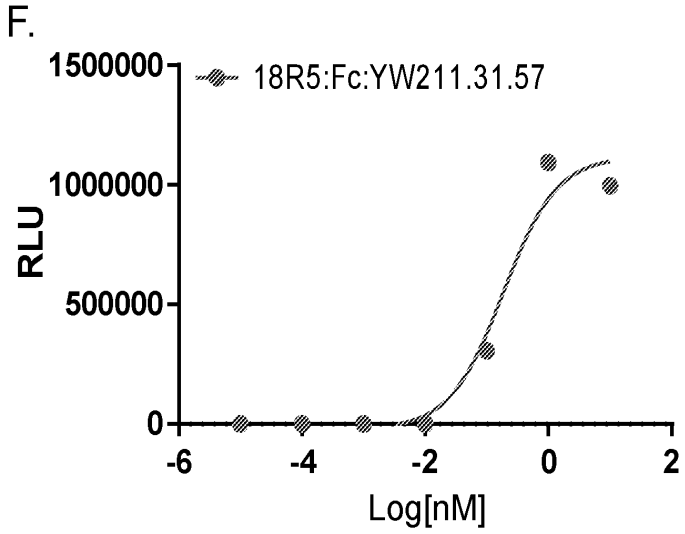

A.
18R5:5:1115.3:Fc
18R5:10:1115.3:Fc
18R5:15:1115.3:Fc
RLU
150000
100000
50000
0
-6
-4
-2
0
2
4
log[nM]
B.
1115.3:5:18R5:Fc
1115.3:10:18R5:Fc
1115.3:15:18R5:Fc
RLU
600000
400000
200000
0
-6
-4
-2
0
2
4
log[nM]
C.
18R5:5:YW211.31.57:Fc
18R5:10:YW211.31.57:Fc
18R5:15:YW211.31.57:Fc
RLU
1500000
1000000
500000
0
-8
-6
-4
-2
0
2
4
log[nM]
D.
YW211.31.57:5:18R5:Fc
YW211.31.57:10:18R5:Fc
YW211.31.57:15:18R5:Fc
RLU
1500000
1000000
500000
0
-8
-6
-4
-2
0
2
4
log[nM]

FIG. 14





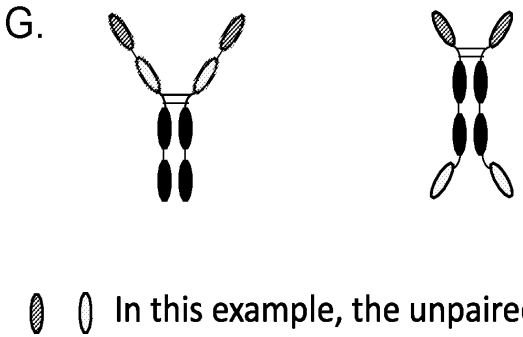


FIG. 14 (contd.)

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H.

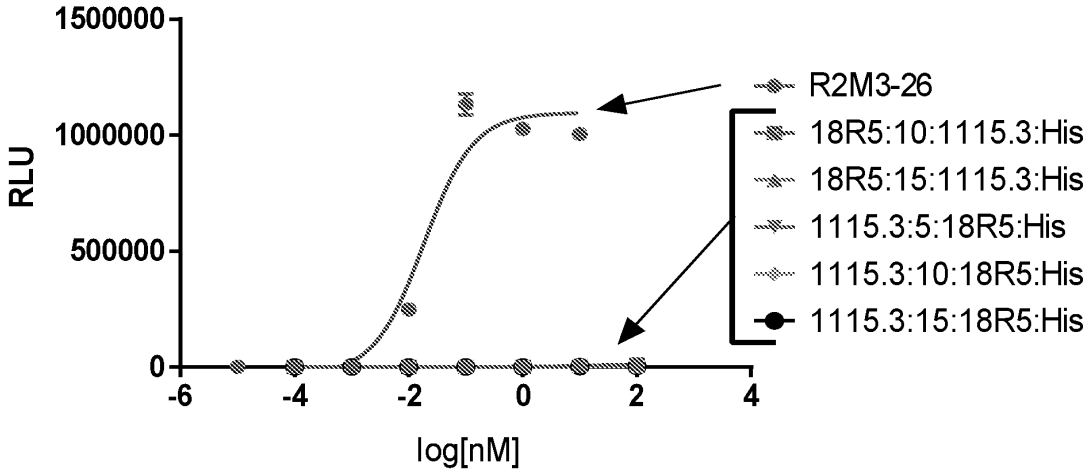


FIG. 14 (contd.)

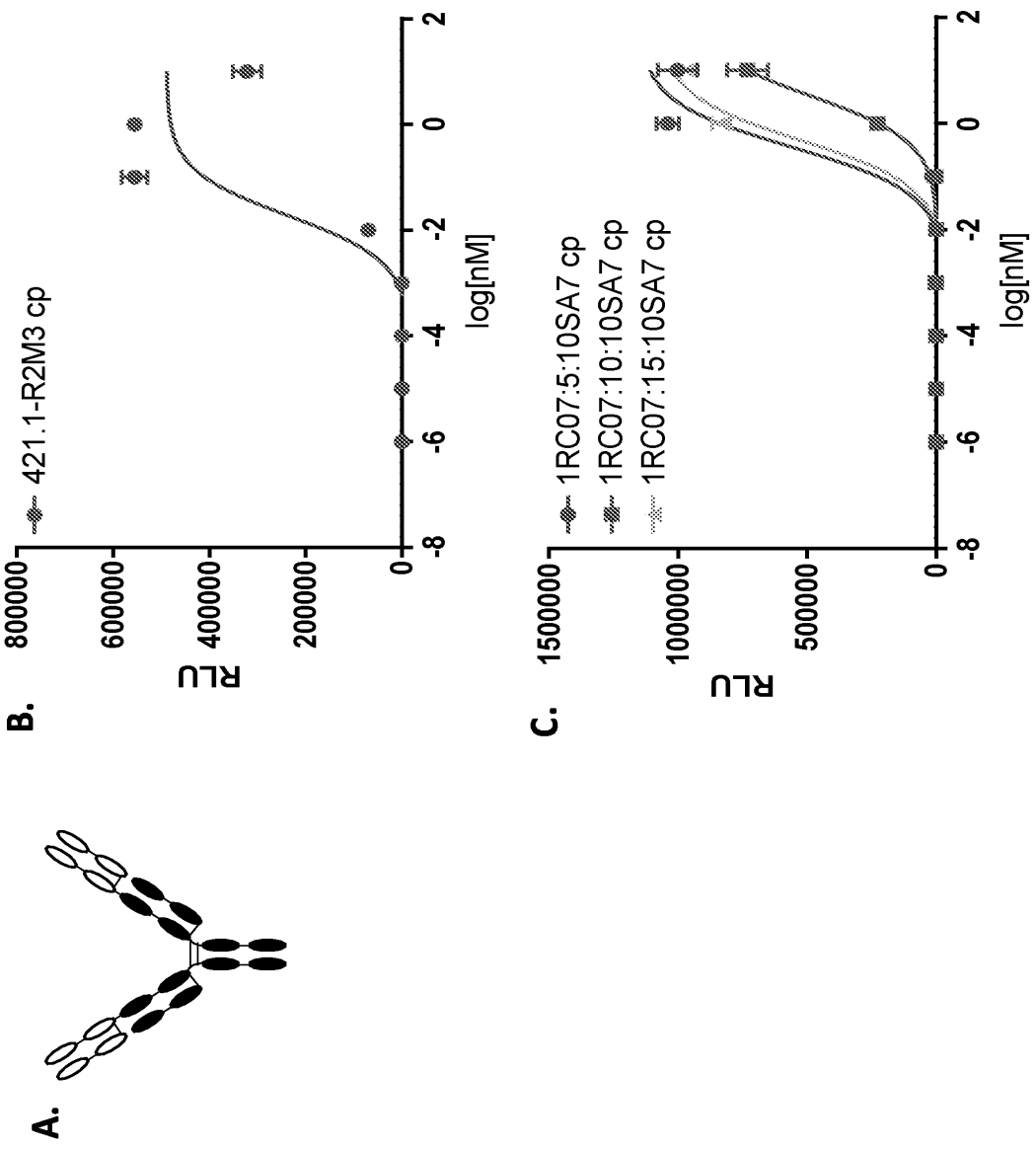

A.
B.
421.1-R2M3 cp
RLU
800000
600000
400000
200000
0
-8
-6
-4
-2
0
2
log[nM]
C.
1RC07:5:10SA7 cp
1RC07:10:10SA7 cp
1RC07:15:10SA7 cp
RLU
1500000
1000000
500000
0
-8
-6
-4
-2
0
2
log[nM]

FIG. 15

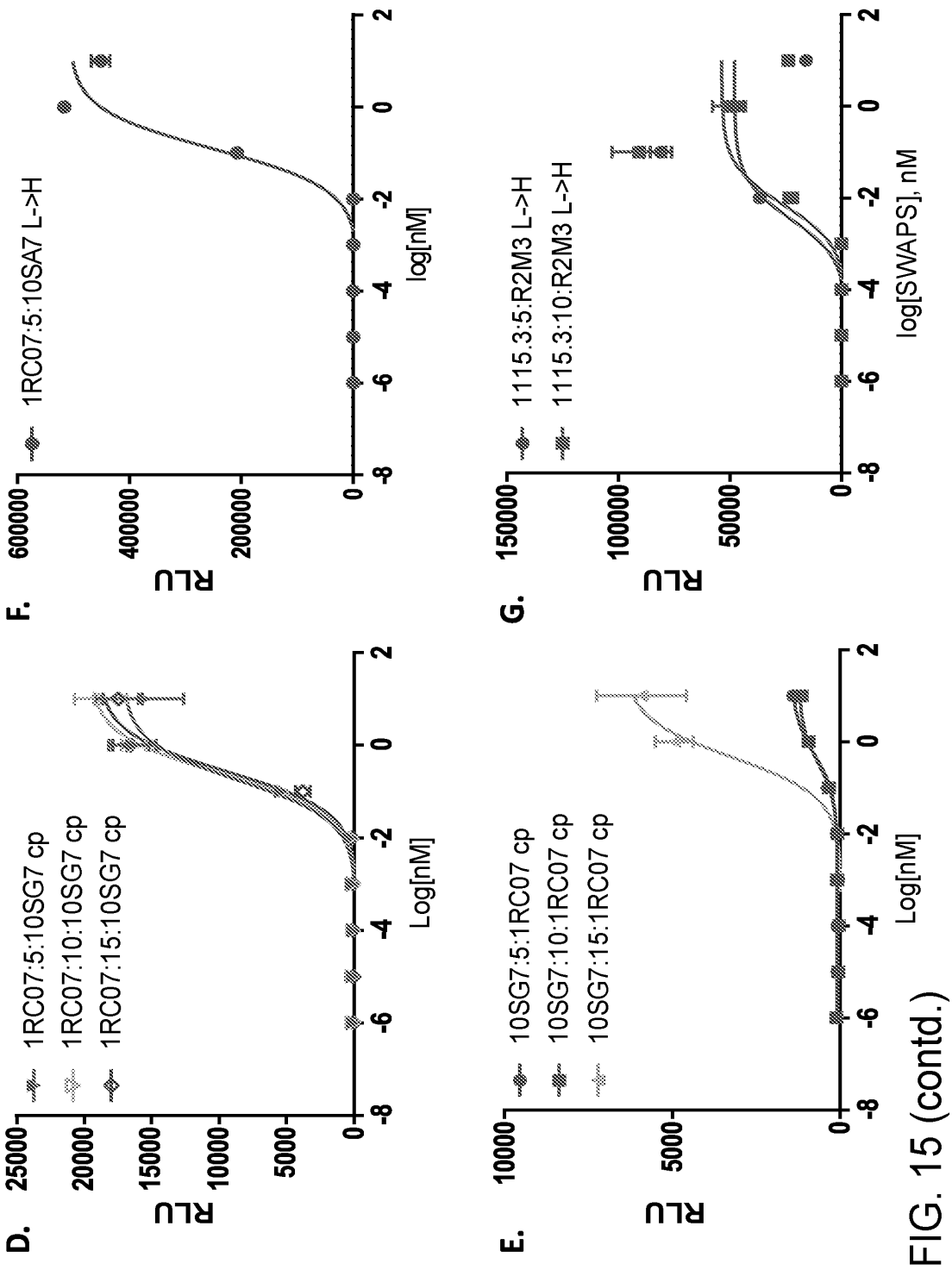

D.
1RC07:5:10SG7 cp
1RC07:10:10SG7 cp
1RC07:15:10SG7 cp
RLU
Log[nM]
F.
1RC07:5:10SA7 L->H
RLU
log[nM]
E.
10SG7:5:1RC07 cp
10SG7:10:1RC07 cp
10SG7:15:1RC07 cp
RLU
Log[nM]
G.
1115.3:5:R2M3 L->H
1115.3:10:R2M3 L->H
RLU
log[SWAPS], nM

FIG. 15 (contd.)

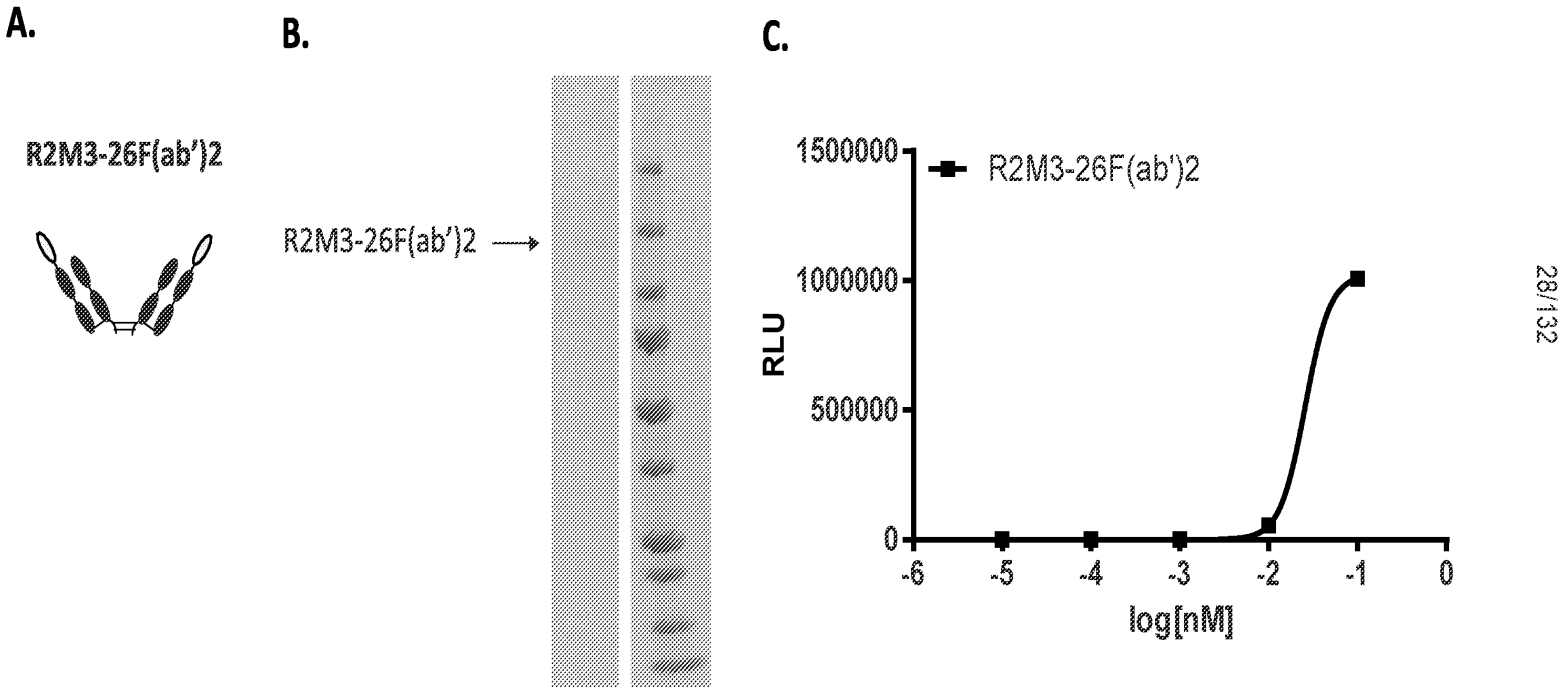

A.
R2M3-26F(ab')2
B.
R2M3-26F(ab')2
C.
R2M3-26F(ab')2
1500000
1000000
500000
0
RLU
-6
-5
-4
-3
-2
-1
0
log[nM]

FIG. 16

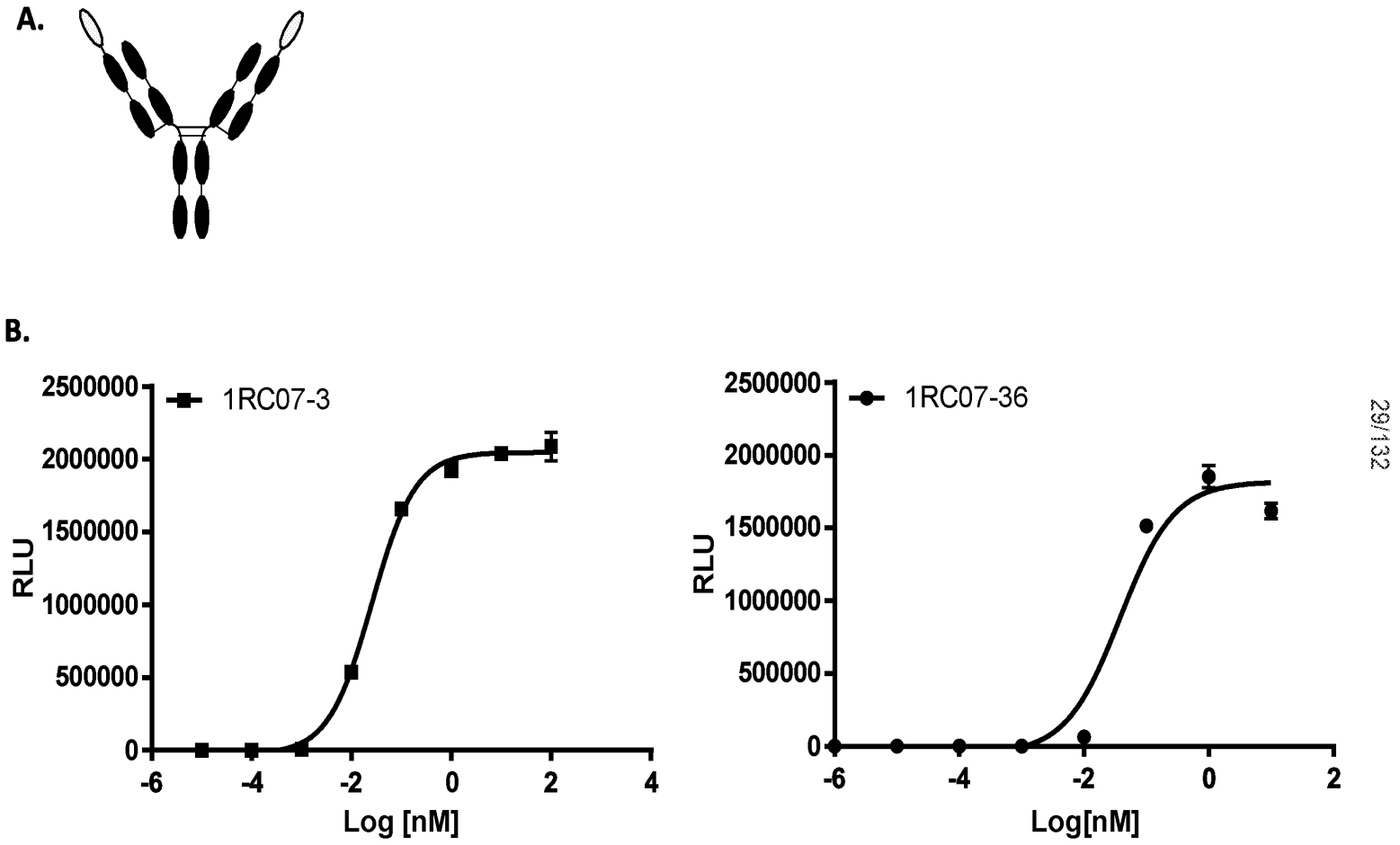
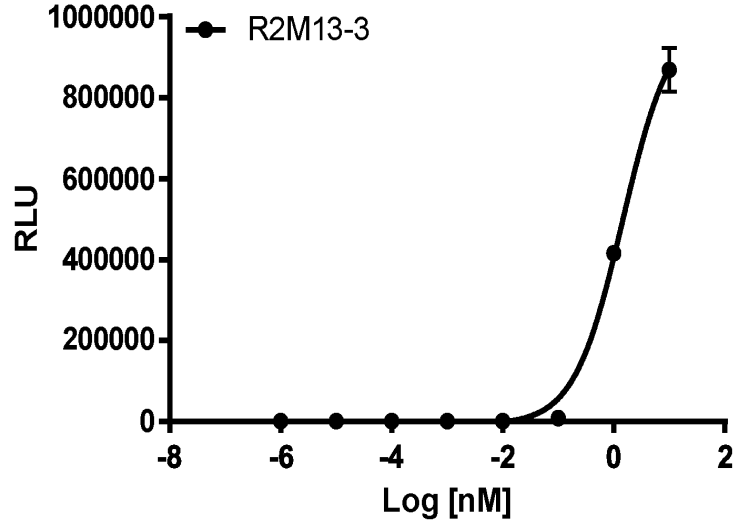
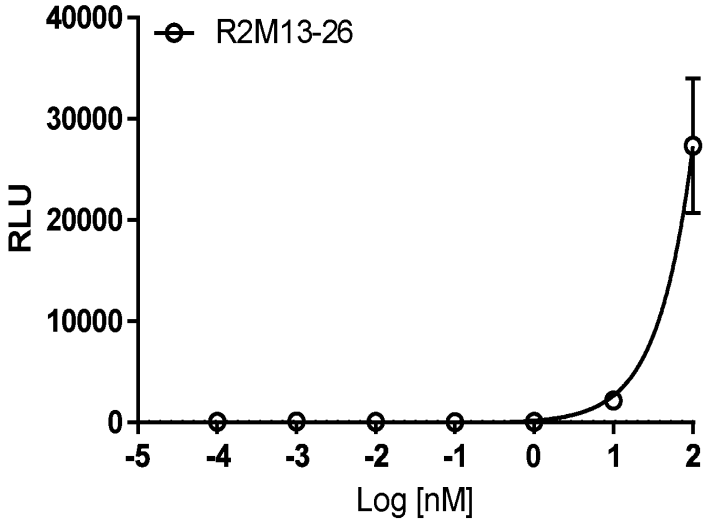


FIG. 17

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C.





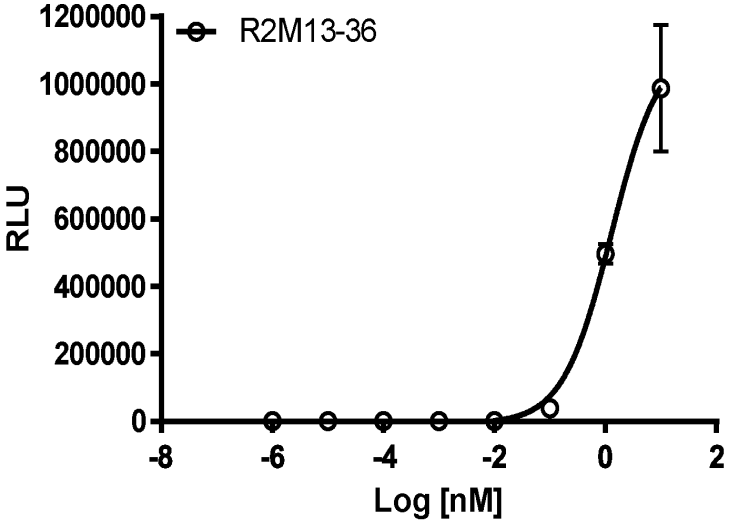


FIG. 17 (contd.)

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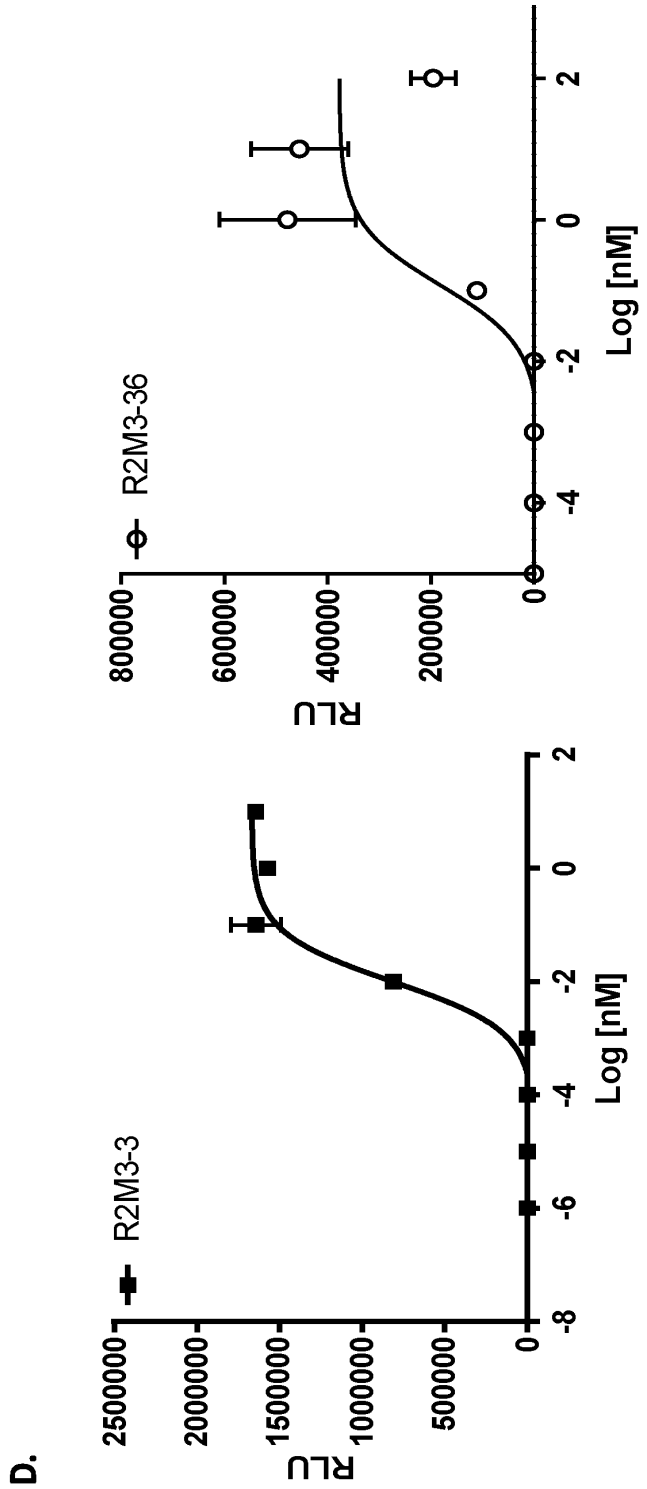

D.
R2M3-3
RLU
2500000
2000000
1500000
1000000
500000
0
-8
-6
-4
-2
0
2
Log [nM]
R2M3-36
RLU
800000
600000
400000
200000
0
-4
-2
0
2
Log [nM]

FIG. 17 (contd.)

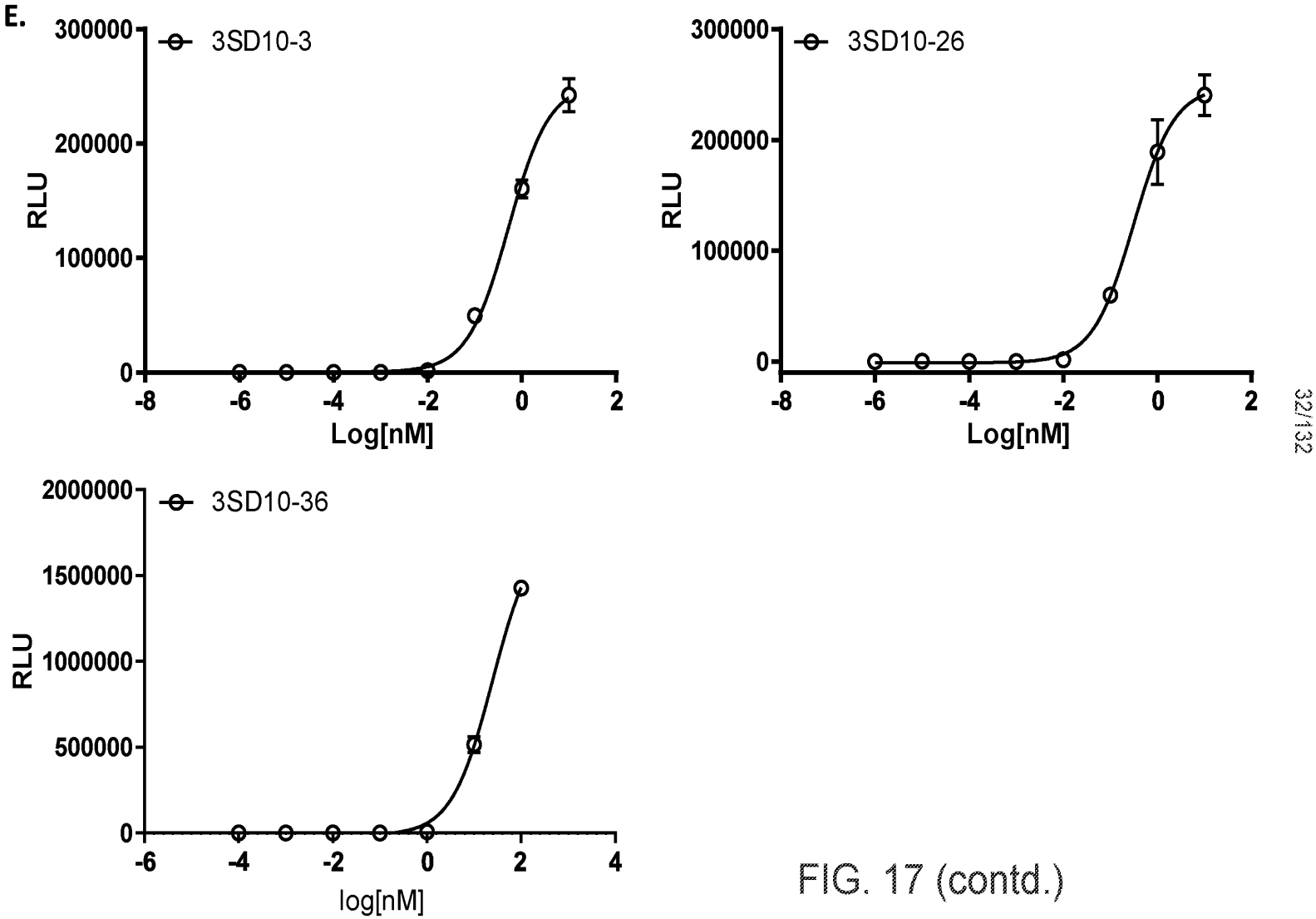


FIG. 17 (contd.)

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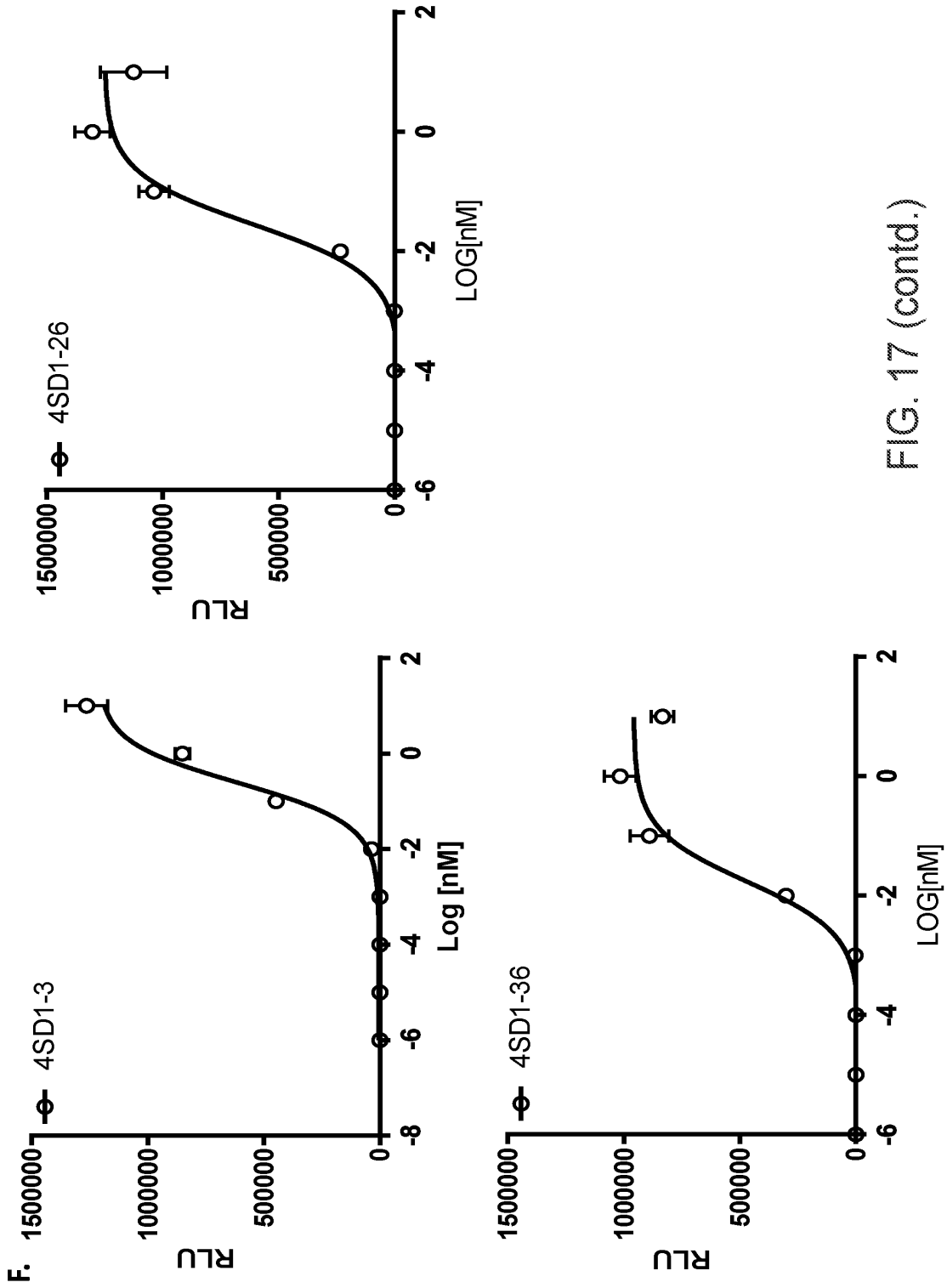

F.
4SD1-3
RLU
1500000
1000000
500000
0
-8
-6
-4
-2
0
2
Log [nM]
4SD1-26
LOG[nM]
4SD1-36

FIG. 17 (contd.)

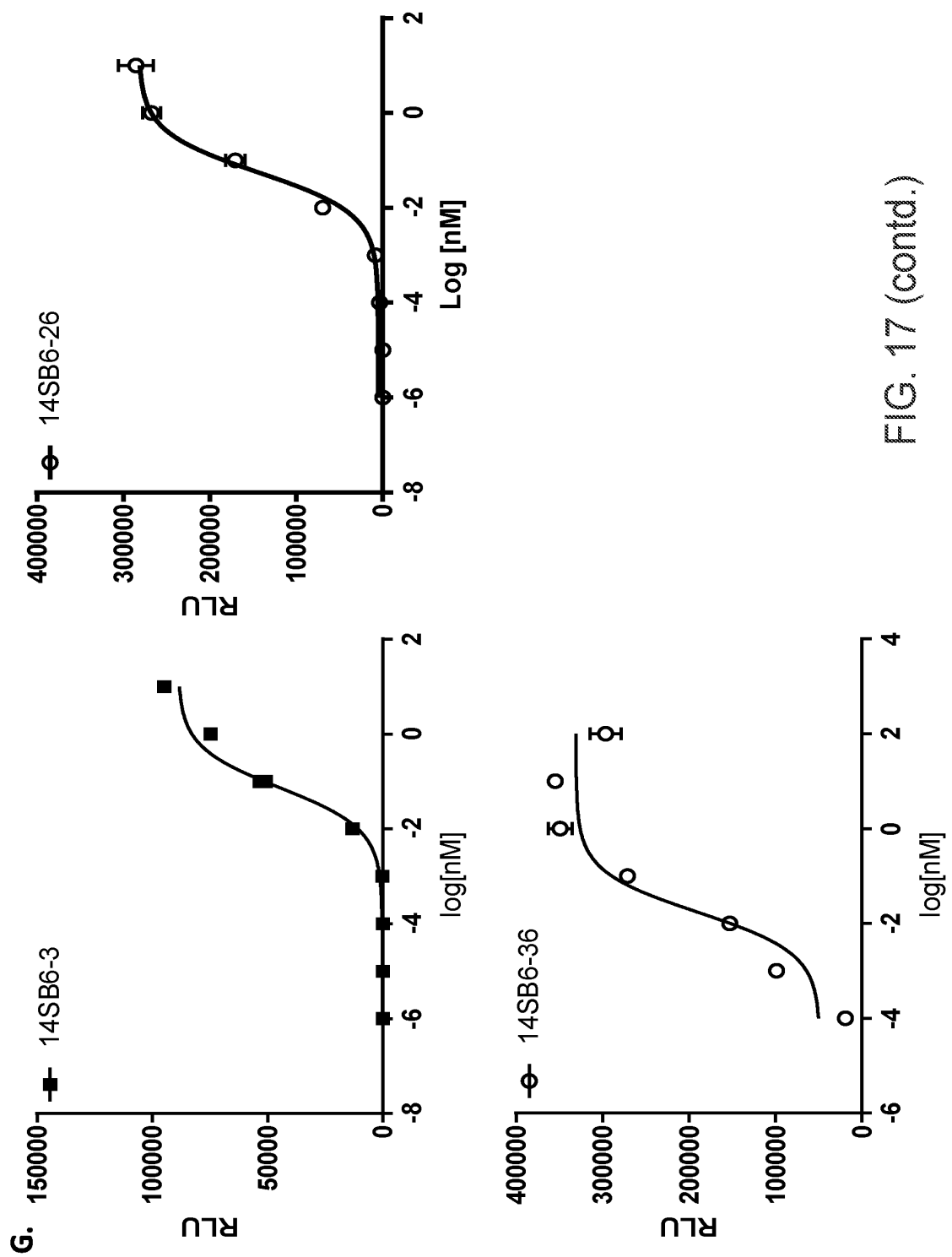

G.
14SB6-3
RLU
150000
100000
50000
0
-8
-6
-4
-2
0
2
log[nM]
14SB6-26
RLU
400000
300000
200000
100000
0
-8
-6
-4
-2
0
2
Log [nM]
14SB6-36
RLU
400000
300000
200000
100000
0
-6
-4
-2
0
2
4
log[nM]

FIG. 17 (contd.)

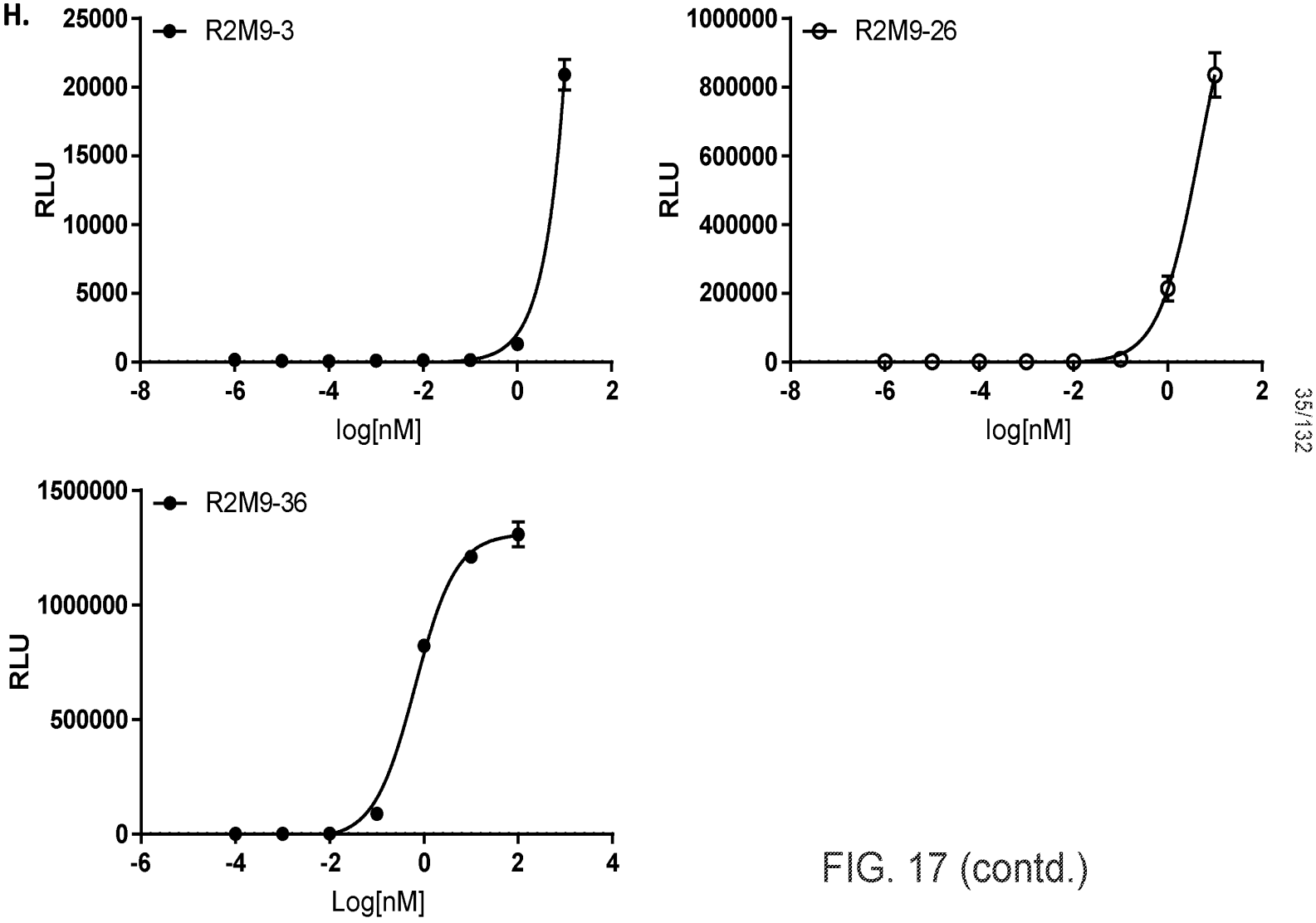


FIG. 17 (contd.)

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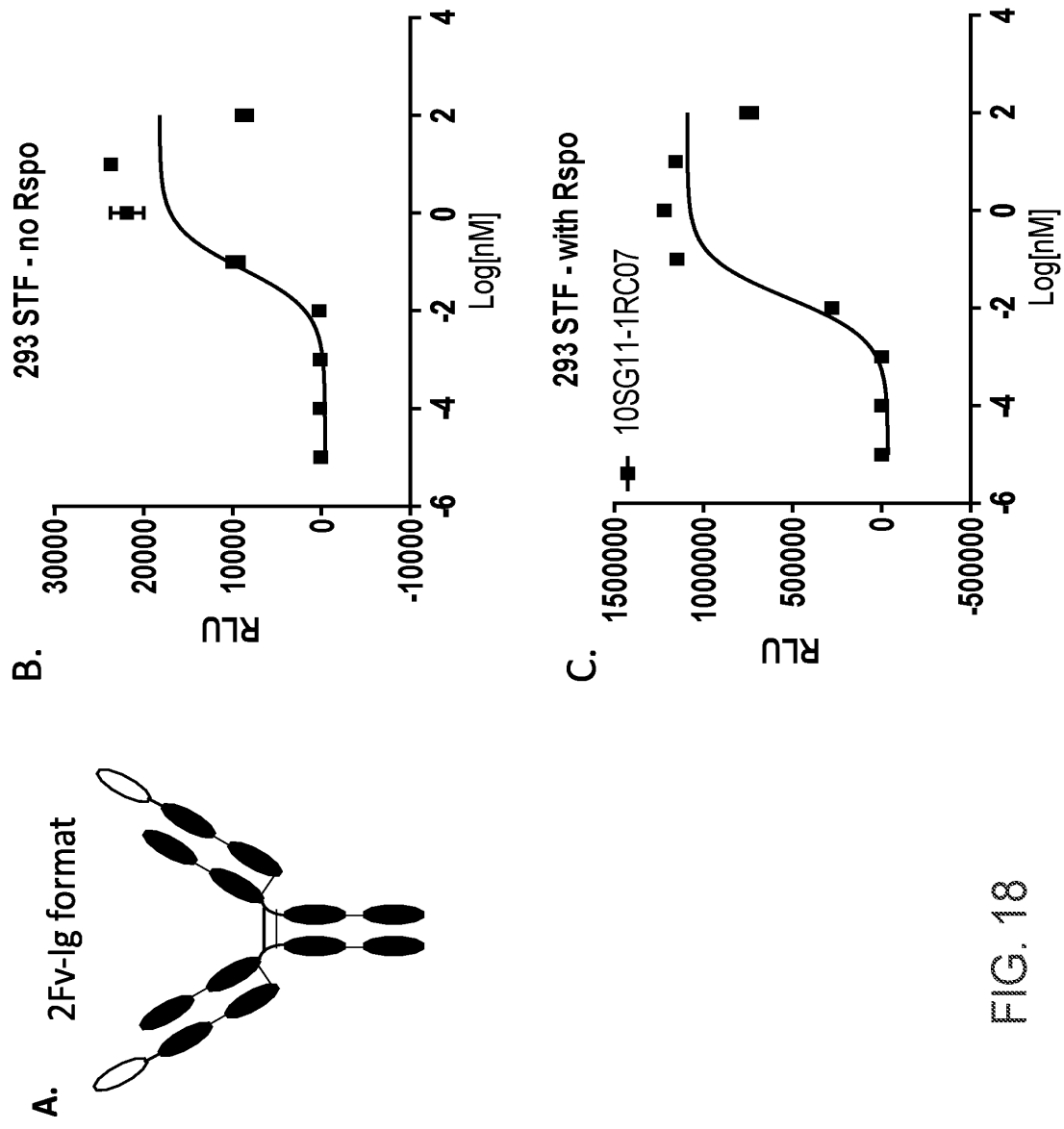

A.
2Fv-Ig format
B.
293 STF - no Rspo
RLU
30000
20000
10000
0
-10000
-6
-4
-2
0
2
4
Log[nM]
C.
293 STF - with Rspo
10SG11-1RC07
RLU
1500000
1000000
500000
0
-500000
-6
-4
-2
0
2
4
Log[nM]

FIG. 18

FIG. 19

Leader = italics
Linker = underlined
VHH/sdAb or VH or VL = bold

*MYRMQLLSCIALSLALVTNS***DVQLVESGGGLVQPGGSLRLSCAASGSIFMINTMAWYRQAPGNQRE**
LVATIRPVVSETTYADAVKGRFTISRDNAKNTVYLQMNSLKSEDTAIYYCNAKRPWGTRDEYWGQG
TLVTVSSGSGGSQTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYYTNT
RSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVTLFP
PSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK
SHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:89)

*MYRMQLLSCIALSLALVTNS***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQREL**
VAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWGQG
TQVTVSSGSGGSQTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYYTNT
RSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVTLFP
PSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK
SHRSYSCQVTHEGSTVEKTVAPTECS* (SEQID NO:90)

*MYRMQLLSCIALSLALVTNS***EVQLVESGGGLVQAGGSLRVSCAASGGTFSRYHMGWFRQAPGKER**
EFVSAITWSGGRTYYADFVKGRFTISRDDARNTVYLQMSSLKPEDTAVYYCALTWAPTPTNRRSDY
AYWGQGTQVTVSSGSGGSQTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPR
TLIYYTNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAA
PSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSL
TPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:91)

*MYRMQLLSCIALSLALVTNS***EVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDMAWYRQAPGNQRE**
LVATIRPVVSETTYADAVKGRFTISRSNAMKTVYLQMNSLKSEDTAIYYCNAKRPWGTRDEYWGQG
TLVTVSSGSGGSQTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYYTNT
RSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVTLFP
PSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK
SHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:92)

*MYRMQLLSCIALSLALVTNS***AVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRQAPGNQREL**
VATIRPVVSETTYADAVKGRFTISRSNAMKTVYLQMNSLKSEDTAIYYCNAKRPWGTRDEYWGQGT
LVTVSSGSGGSQTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYYTNTR
SSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVTLFPP
SSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKS
HRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:93)

*MYRMQLLSCIALSLALVTNS***AVQLVDSGGGLVQAGGSLRLSCAVSGRTFSMYDMGWFRQAPGKER**
EFVASIRWSSGNTWYADSMKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCYANIYYTRRAPEEYW
GQGTLVTVSSGSGGSQTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIY
YTNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSV
TLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPE
QWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:94)

*MYRMQLLSCIALSLALVTNS*EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEW
MGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYGMDVW
GQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK* (SEQ ID NO:95)

FIG. 19 (contd.)

*MYRMQLLSCIALSLALVTNS***EVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQRELI ANMRGGGYMKYAGSLKGRFTMSTESAKNTLYLQMNSLKPEDTAVYYCYARTQRMGVVNSYWGQ GTLVTVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRP SGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPS SEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSH RSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:96)

*MYRMQLLSCIALSLALVTNS***QVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQREL IANMRGGGYMKYAGSLKGRFTMSTESAKNTLYLQMNSLKPEDTAVYFCNAVTYDGYTIRGQGTLVT VSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPER FSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQA NKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQ VTHEGSTVEKTVAPTECS* (SEQ ID NO:97)

*MYRMQLLSCIALSLALVTNS***EVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQRELI ANMRGGGYMKYADSVQGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAQFRNDYGLRYQSTNN YWGQGTLVTVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDK SNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTL FPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQ WKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:98)

*MYRMQLLSCIALSLALVTNS***QVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQREL IANMRGGGYMKYAGSLKGRFTMSTESAKNTVYLQMNSLKPEDSAVYYCNANYRGNRYWGQGTLV TVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPE RFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQ ANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSC QVTHEGSTVEKTVAPTECS* (SEQ ID NO:99)

*MYRMQLLSCIALSLALVTNS***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQREL VAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWGQG TQVTVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPS GIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPSS EELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHR SYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:100)

*MYRMQLLSCIALSLALVTNS***EVQLVESGGGLVQAGGSLRVSCAASGGTFSRYHMGWFRQAPGKER EFVSAITWSGGRTYYADFVKGRFTISRDDARNTVYLQMSSLKPEDTAVYYCALTWAPTPTNRRSDY AYWGQGTQVTVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIY DKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSV TLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPE QWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:101)

*MYRMQLLSCIALSLALVTNS***AVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRQAPGNQREL VATIRPVVSETTYADAVKGRFTISRSNAMKTVYLQMNSLKSEDTAIYYCNAKRPWGTRDEYWGQGT LVTVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGI PERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFPPSSEE LQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSY SCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:102)

*MYRMQLLSCIALSLALVTNS***AVQLVDSGGGLVQAGGSLRLSCAVSGRTFSMYDMGWFRQAPGKER EFVASIRWSSGNTWYADSMKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCYANIYYTRRAPEEYW GQGTLVTVSS**GSGGSDIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSN RPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGQPKAAPSVTLFP PSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:103)

FIG. 19 (contd.)

*MYRMQLLSCIALSLALVTNS***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLE WVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGT LVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG K* (SEQ ID NO:104)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGDVVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKPGQSPQLLI YLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQGTHWPYTFGQGTKLEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:105)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGDIVMTHTPLSLSVTPGEPASISCRSSRSLLDTDDGNTYLDWYLQKPGQSPQLLI HTLSHRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQSIQLPWTFGQGTKVEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:106)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGSYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQQKPGQSPVLVIYEDSQ RPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQPKAAPSVTLF PPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQW KSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:107)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGQSVVTQPPSVSGAPGQRVTISCTRSSSNIGAGYDVHWYQQHPGTAPKLLIFG NSIRPSGVPDRFSGSKSGTSATLGITGLQTGDEADYYCGTWDSSLSAWVFGGGTKLTVLGQPKAAPS VTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTP EQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:108)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGEIVLTQSPATLSLSPGEGATLSCRASQSVGTYLTWYRQKPGQAPRLLIYDASN RATGIPARFSGSGAGTDFTLKISRVEAEDVGVYYCMQATQFPLTFGQGTKVEIKRTVAAPSVFIFPPSD EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKH KVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:109)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGDIQLTQSPSSLSASVGDRVTITCRASRSISSYFNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQADTFPPTFGQGTRLEIKRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:110)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQESGPGLVKPSETLSLTCTVSGGSISGNNYYWGWIRQPPG KGLEWIGSIYFTGGTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARVMLITDAFDIWGQ GTMVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFP PKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV

FIG. 19 (contd.)

EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GKGSGSGHHHHHH** (SEQ ID NO:111)

*MDMRVPAQLLGLLLLWLRGARC***QLQLQESGSGLVKPSQTLSLTCAVSGGSISSGGYSWSWIRQPP**
GKGLEWIGSIYHSGSTYYNPSLKSRLTISVDTSKNQFSLKLSSVTAADTAVYYCARFYYDILTGYSYF
DYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF
PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGK153HHHHHH** (SEQ ID NO:112)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRQPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPGKGSGSGHHHHHH* (SEQ ID NO:113)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQHPG**
KGLEWIGYIYYSGSTYYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCARATYGGDAFDIWG
QGTMVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
SSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLF
PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH
QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGKGSGSGHHHHHH** (SEQ ID NO:114)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQESGPGLVKPSETLSLTCTVSGGAISGTSYFWGWIRQPPG**
KGLEWIGSIYYTGNTYYNPSLKSRLTVSVDTSKNQFSLNLNSVTAADTATYYCARIGIAVAAPVDHW
GQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGKGSGSGHHHHHH* (SEQ ID NO:115)

*MDMRVPAQLLGLLLLWLRGARC***QLQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPG**
KGLEWIGYIYYSGSTYYNPSLKSRVTISVDKSKNQFSLKLSSVTAADTAVYYCARVRDYYDSSGYYY
DYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELL
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY
TQKSLSLSPGKGSGSGHHHHHH** (SEQ ID NO:116)

FIG. 19 (contd.)

MDMRVPAQLLGLLLLWLRGARCEVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQG
LEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYGMD
VWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGK<u>GSGSG</u>**DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQRELVAMIRP
VVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWGQGTQVTVS
S*** (SEQ ID NO:117)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSS**<u>GSGSG</u>EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWI
SAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYGMDVWGQGTT
VTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL
YSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL
NGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*
(SEQ ID NO:118)

*MDMRVPAQLLGLLLLWLRGARC*QAVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQ
APRTLIYYTNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQP
KAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSY
LSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS<u>GSGSG</u>**DVQLVESGGGLVQAGGSLRLACAGS
GRIFAIYDIAWYRHPPGNQRELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAV
YYCNAKRPWGSRDEYWGQGTQVTVSS*** (SEQ ID NO:119)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSS**<u>GSGSG</u>QAVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYY
TNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVT
LFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQ
WKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:120)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQ
GLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYG
MDVWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC<u>GSGSG</u>HHHHHH* (SEQ
ID NO:122)

*MYRMQLLSCIALSLALVTNS***AVQLVDSGGGLVQAGGSLRLSCAVSGRTFSMYDMGWFRQAPGKER
EFVASIRWSSGNTWYADSMKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCYANIYYTRRAPEEYW
GQGTLVTVSS<u>GSGGS</u>QTVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIY
YTNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSV
TLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPE
QWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:123)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQ
GLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYG
MDVWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC<u>GSGSG</u>HHHHHH* (SEQ
ID NO:124)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***QAVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPG**
QAPRTLIYYTNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLG
QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAS
SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:125)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQ**
GLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYG
MDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY
TQKSLSLSPGK* (SEQ ID NO:126)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTV
DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGSGSGHHHHHH* (SEQ ID NO:127)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV
DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGS**AVQQVESGGGLVQPGGSLRLSCAA**
SGFTLDYYAISWFRQAPGKKRELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTA
VYFCNAVTYNGYTIWGQGTQVTVSS*** (SEQ ID NO:128)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ**
RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ
GTQVTVSSGSGSGSYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQQKPGQSPVLVIYEDSQR
PSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQPKAAPSVTLFP
PSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWK
SHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:134)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK**
EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV
SSGSGSGSYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQQKPGQSPVLVIYEDSQRPSGIPVR
FSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQ
ANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSC
QVTHEGSTVEKTVAPTECS* SEQ ID NO:135)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ**
RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ
GTQVTVSSGGSGSGSGDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAA
SSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE
KHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:136)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSGSGSGDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL
QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK
VYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:137)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK**
EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV
SSGSGSGDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA
SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT
HQGLSSPVTKSFNRGEC* (SEQ ID NO:138)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ**
RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ
GTQVTVSSGSGSGQAVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYYT
NTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVTL
FPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQ
WKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:139)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK**
EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV
SSGSGSGQAVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQQTPGQAPRTLIYYTNTRSSDV
PERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVLGQPKAAPSVTLFPPSSEEL
QANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYS
CQVTHEGSTVEKTVAPTECS* (SEQ ID NO:140)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ**
RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ
GTQVTVSSGGSGSDIQMTQSPSSLSASVGDRVTITCRASQGISSYLAWYQQKPGKAPKLLIYAASNLL
GGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYSTPWTFGQGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK
VYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:141)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSGSGGSDIQMTQSPSSLSASVGDRVTITCRASQGISSYLAWYQQKPGKAPKLLIYAASNL
LGGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYSTPWTFGQGTKVEIKRTVAAPSVFIFPPSD
EQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKH
KVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:142)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK**
EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV
SSGGSGSDIQMTQSPSSLSASVGDRVTITCRASQGISSYLAWYQQKPGKAPKLLIYAASNLLGGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQTYSTPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGT
ASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEV
THQGLSSPVTKSFNRGEC* (SEQ ID NO:143)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ**
RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ
GTQVTVSSGGSGSDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQ
SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQ
LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV
YACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:144)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSGGSGSDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL
QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK
VYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:145)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV SS**GGSGSDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC* (SEQ ID NO:146)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ GTQVTVSS**GGSGSDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQ SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:147)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GGSGSDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:148)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV SS**GGSGSDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC* (SEQ ID NO:149)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQ RELIANMRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYVKLRDDDYVYRGQ GTQVTVSS**GSGSGDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQ SGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYRTPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQ LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:150)

M*DMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**GSGSGDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSL QSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYRTPFTFGPGTKVDIKRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:151)

*MDMRVPAQLLGLLLLWLRGARC***QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGK EREFVSGIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSRGYWGQGTLVTV SS**GSGSGDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSR FSGSGSGTDFTLTISSLQPEDFATYYCQQSYRTPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQLKSGTA SVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC* (SEQ ID NO:152)

*MDMRVPAQLLGLLLLWLRGARC***EVQLLQSGAEVKKPGSSVKVSCKASGGTFTYRYLHWVRQAPGQ GLEWMGGIIPIFGTGNYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCASSMVRVPYYYGMD VWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPS VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK SLSLSPGK* (SEQ ID NO:153)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVKPGGSLRLSCAASGFNFGIYSMTWVRQAPGK GLEWISYISGDSGYTNYADSVKGRFTISRDDSKNTLYLQMNSLKTEDTAVYYCARVGPGGWFDPWG QGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ SSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLF PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PGK* (SEQ ID NO:154)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVKPGGSLRLSCAASGFTFTNYAMSWVRQAPGK GLEWVSAISGSGGSTYYADSVKGRFTISRDDSKNTLYLQMNSLKTEDTAVYYCARATGFGTVVFDY WGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS LSLSPGK* (SEQ ID NO:155)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVQSGGGLVKPGGSLRLSCAASGFTFSSYSMNWVRQAPG KGLEWVSYIENDGSITTYADSVKGRFTISRDDSKNTLYLQMNSLKTEDTAVYYCARAPYYYGSGSLF RLDYWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK* (SEQ ID NO:156)

*MDMRVPAQLLGLLLLWLRGARC***QVQLVQSGAEVKKPGSSVKVSCKASGYTFTNNFMHWVRQAPG QGLEWMGWINPNSGGTKYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARSVGEVGATM LGIGVWYWFDPWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK* (SEQ ID NO:157)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG QGTQVTVSS**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGS**AVQQVESGGGLVQPGGSLRLSCAA SGFTLDYYAISWFRQAPGKKRELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTA VYFCNAVTYNGYTIWGQGTQVTVSS**GSGSGHHHHHH* (SEQ ID NO:2192)

*MDMRVPAQLLGLLLLWLRGAR***AVQQVESGGGLVQPGGSLRLSCAASGFTLDYYAISWFRQAPGKK RELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYFCNAVTYNGYTIWGQGTQ VTVSS**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVY TLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGS**DVQLVESGGGLVQAGGSLRLACAGSGRIF AIYDIAWYRHPPGNQRELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYC NAKRPWGSRDEYWGQGTQVTVSS*** (SEQ ID NO:2193)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSGGSGS**AVQQVESGGGLVQPGGSLRLSCAASGFTLDYYAISWFRQAPGKKRELVADI**
TSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYFCNAVTYNGYTIWGQGTQVTVSSDK
THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT
KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRE
EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2194)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSGSSGSGGGSGS**AVQQVESGGGLVQPGGSLRLSCAASGFTLDYYAISWFRQAPGKKR**
ELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYFCNAVTYNGYTIWGQGTQV
TVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE
VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYT
LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2195)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSGSSGSGGGGSGGSGS**AVQQVESGGGLVQPGGSLRLSCAASGFTLDYYAISWFRQA**
PGKKRELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYFCNAVTYNGYTIWG
QGTQVTVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV
DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2196)

*MDMRVPAQLLGLLLLWLRGARC***AVQQVESGGGLVQPGGSLRLSCAASGFTLDYYAISWFRQAPGK**
KRELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYFCNAVTYNGYTIWGQGT
QVTVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQV
YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGS**DVQLVESGGGLVQAGGSLRLACAGSGR**
IFAIYDIAWYRHPPGNQRELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYY
CNAKRPWGSRDEYWGQGTQVTVSS* (SEQ ID NO:2197)

*MDMRVPAQLLGLLLLWLRGARC***DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQ**
RELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNAKRPWGSRDEYWG
QGTQVTVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV
DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGS**DVQLVESGGGLVQAGGSLRLACAG**
SGRIFAIYDIAWYRHPPGNQRELVAMIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTA
VYYCNAKRPWGSRDEYWGQGTQVTVSS* (SEQ ID NO:2198)

*MDMRVPAQLLGLLLLWLRGARC***AVQQVESGGGLVQPGGSLRLSCAASGFTLDYYAISWFRQAPGK**
KRELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYFCNAVTYNGYTIWGQGT
QVTVSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQV
YTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSGS**AVQQVESGGGLVQPGGSLRLSCAASGF**
TLDYYAISWFRQAPGKKRELVADITSGGSTNYADSVKGRFTISRDNAKNTIYLQMNSLKPEDTAVYF
CNAVTYNGYTIWGQGTQVTVSSGSGSGHHHHHH* (SEQ ID NO:2199)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK**
GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG
QGTLVTVSSGGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ**
APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLG
SGSG**DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEKRLELVAAINLNGGSTYYS**
DTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYWGQGTLVTVSAGSAASGS
SGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHESPRLLIKYTSQSISGIPS**
RFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGSGSGDKTHTCPPCPAPEA
AGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV
KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH
YTQKSLSLSPGK* (SEQ ID NO:2200)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK**
GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG
QGTLVTVSSGGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ**
APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLG
SGSGGSGSG**DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEKRLELVAAINLNGG**
STYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYWGQGTLVTVSAGS
AASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHESPRLLIKYTSQSI**
SGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGSGSGDKTHTCPPC
PAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH
EALHNHYTQKSLSLSPGK* (SEQ ID NO:2201)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK**
GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG
QGTLVTVSSGGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ**
APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLG
SGSGGSGSGGSSGG**DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEKRLELVAAI**
NLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYWGQGTLVT
VSAGSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHESPRLLIK**
YTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGSGSGDKTH
TCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEM
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2202)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK**
RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW
GQGTLVTVSAGSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH**
ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGS
GSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGSYTYYAD**
SVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSSGGGGSGGGG
SGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPERF**
SGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLGSGSGDKTHTCPPCPAPEAA
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY
TQKSLSLSPGK* (SEQ ID NO:2203)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**GS GSGGSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGS YTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS**GGGG SGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPS GIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**DKTHTCPPCP APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN STYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA LHNHYTQKSLSLSPGK* (SEQ ID NO:2204)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**GS GSGGSGSGGSSGG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVI SGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVS S**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYD KSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**DKTH TCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEM TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2205)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEISPYSGSTYYAD SVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWGQGTLVTVSS**GS AASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASF LYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIK**GSGSG**DKTHTCPP CPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK* (SEQ ID NO:2206)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSGGSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEISPYSG STYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWGQGTLV TVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLL IYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIK**GSGSG**DKT HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2207)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSGGSGSGGSSGG**EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEI SPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWG QGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIK**GS GSG**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2208)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGD GSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS**GG GGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNR PSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**DKTHTCPP CPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPGK* (SEQ ID NO:2209)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSGGSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVS VISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTV SS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIY DKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**DKT HTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREE MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2210)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSGGSGSGGSSGG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSG**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2211)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSGDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK**GSGSG**DVKLVESGGGLVKLGGSLRLSCAASGFSFS TSYMSWVRQTPEKRLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYC ASELAGYGTPFAYWGQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQ SISYNLHWYQQKSHESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWP LTFGAGTKLEVK* (SEQ ID NO:2212)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**GS GSGDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK**GSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSH YTLSWVRQAPGKGLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCA RNFIKYVFANWGQGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSF YVHWYQQKPGQAPVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLV FGGGTKLTVL* (SEQ ID NO:2213)

*MDMRVPAQLLGLLLLWLRGARC***QAVVLQEPSLSVSPGGTVTLTCGLSSGSVSTNYYPSWYQDTPG QDPRTLIYYTNTRSSDVPERFSGSIVGNKAALTITGAQPDDESVYFCLLYLGRGIWVFGGGTKLTVL**G QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAK SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2214)

*MDMRVPAQLLGLLLLWLRGARC***QIVLTQSPAIMSASPGEKVTISCSANSSVRFMFWYQKKPGSKPK PLIYRTSNLASGVPARFSGSGSGTSYSLTISSMEAEDAATYYCQQYHSYPWTFGGGTKLEIK**RTVAA PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTL TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2215)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQSGPELVKPGASVRISCKASGYTFTTYYIHWLKDRPGQG LEWIGWIFPGNVNTKYNAKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCAREELQYYFDYWGD GSTLTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLKSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC**GSGSG**EVQLVQSGAEVKKPGAS VKVSCKASGYTFTSYGISWVRKAPGQGLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYME LRSLRSDDTAVYYCASSKEKATYYYGMDVWGKGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKV DKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTV DKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2216)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVL**GQ PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2217)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQKKPGKKP KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIK**RTVAA PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTL TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2218)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSG**EVQLVESGGG**
LVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGKGLEWVASISSTSGSKYYADSVKGRFTISRDNS
KNTLYLQMNSLRAEDTAVYSCAKTYYDFWSGYYTFDYWGDGTLVTVSSASTKGPSVFPLAPSSKST
SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTIS
KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF
FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2219)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2220)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2221)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSGGSGSG**EVQLV**
ESGGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGKGLEWVASISSTSGSKYYADSVKGRFTI
SRDNSKNTLYLQMNSLRAEDTAVYSCAKTYYDFWSGYYTFDYWGDGTLVTVSSASTKGPSVFPLAP
SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTVPSSSLGTQT
YICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAP
IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2222)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2223)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAA
PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2224)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSGGSGSGGSSGG
EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGKGLEWVASISSTSGSKYYADSVK
GRFTISRDNSKNTLYLQMNSLRAEDTAVYSCAKTYYDFWSGYYTFDYWGDGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2225)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2226)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSAYVGDRVTITCRVSQGISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYTIPFTFGPGTKVDIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2227)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSG**EVQLVESVGG**
LVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGKGLEWISTISGSGGSTYYADSVKGRFTISRDNSK
NTLYLQMNSLRAEDTAVYYCARAGYGRYYYGMDVWGDGTTVTVSSASTKGPSVFPLAPSSKSTSG
GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2228)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2229)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSAYVGDRVTITCRVSQGISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYTIPFTFGPGTKVDIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2230)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSGGSGSG**EVQLV**
ESVGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGKGLEWISTISGSGGSTYYADSVKGRFTIS
RDNSKNTLYLQMNSLRAEDTAVYYCARAGYGRYYYGMDVWGDGTTVTVSSASTKGPSVFPLAPSS
KSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTVPSSSLGTQTYIC
NVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIE
KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2231)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2232)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSAYVGDRVTITCRVSQGISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYTIPFTFGPGTKVDIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2233)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGK**
GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH
GLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSGGSGSGGSSGG
EVQLVESVGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGKGLEWISTISGSGGSTYYADSVK
GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAGYGRYYYGMDVWGDGTTVTVSSASTKGPSVF
PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLKSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2234)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSAYVGDRVTITCRVSQGISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYTIPFTFGPGTKVDIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2235)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2236)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESVGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGK**
GLEWISTISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAGYGRYYYGMD
VWGDGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSG**QVQLQQWGAGLLK**
PSETLSLTCAVSGASFSGHYWTWIRKPPGKGLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSL
NLKSVTAADTAVYYCARGGQGGYDWGHYHGLDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGG
TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLESVVTVPSSSLGTQTYICNVNHKP
SNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY
SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2237)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSAYVGDRVTITCRVSQGISSYLNWYQKKPGKKP**
KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYTIPFTFGPGTKVDIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2238)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP**
VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQ
PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS
YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2239)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESVGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGK**
GLEWISTISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAGYGRYYYGMD
VWGDGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCGSGSGGSGSG**QVQLQQWG**
AGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGKGLEWIGEIDHTGSTNYEPSLRSRVTISVDTSK
NQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYHGLDVWGKGTTVTVSSASTKGPSVFPLAPSSK
STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLESVVTVPSSSLGTQTYICN
VNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEK
TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD
GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2240)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSAYVGDRVTITCRVSQGISSYLNWYQKKPGKKP KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTYTIPFTFGPGTKVDIK**RTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLESTLTL SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2241)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQDKPGQDP VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVL**GQ PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAKS YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS* (SEQ ID NO:2242)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESVGGLVQPGGSLRLSCAASGFTFSSYAMHWVRDAPGK GLEWISTISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAGYGRYYYGMD VWGDGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLKSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC<u>GSGSGGSGSGGSSGG</u>**QVQ LQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRKPPGKGLEWIGEIDHTGSTNYEPSLRSRVTIS VDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYHGLDVWGKGTTVTVSS**ASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLESVVTVPSSSLGT QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALG APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2243)

*MDMRVPAQLLGLLLLWLRGARC***QVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRQPPGK GLEWIGEIDHTGSTNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYH GLDVWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC<u>GSGSG</u>HHHHHH* (SEQ ID NO:2244)

*MDMRVPAQLLGLLLLWLRGARC***DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAP KLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIK**RTVAA PSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTL TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC* (SEQ ID NO:2245)

*MDMRVPAQLLGLLLLWLRGARC***SYVLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQQKPGQSP VLVIYEDSQRPSGIPVRFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVL**GQ PKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASS YLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS<u>GSGSG</u>**EVQLVESGGGLVQPGGSLRLSCAA SGFTFSSYAMHWVRQAPGKGLEWVASISSTSGSKYYADSVKGRFTISRDNSKNTLYLQMNSLRAE DTAVYSCAKTYYDFWSGYYTFDYWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP KSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK* (SEQ ID NO:2246)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC<u>GSGSG</u>HHHHHH* (SEQ ID NO:2247)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC*AAA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHE SPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**RTV AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*GGGGS***EVQLVQSGAEVKKPGASVKVSCK ASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRS DDTAVYYCASSKEKATYYYGMDVWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP KSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGK*GSGSG* HHHHHH* (SEQ ID NO:2248)

*MDMRVPAQLLGLLLLWLRGARC*AAA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHE SPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**RTV AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSS TLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*GGGGSGGGGS***EVQLVQSGAEVKKPGASV KVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMEL RSLRSDDTAVYYCASSKEKATYYYGMDVWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD KKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*GSGSG*HHHHHH* (SEQ ID NO:2249)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS***GGGGSGGGGSGGGGS***DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL*G* SGSGDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYT LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK*GSGSG****EVQLVESGGGLVQPGGSLRLSCAASGFTFT SYYISWVRQAPGKGLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC ALRARPPIRLHPRGSVMDYWGQGTLVTVSS***GSAASGSSGGSSSGA***DIQMTQSPSSLSASVGDRVTI TCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQSYTTPPTFGQGTKVEIK*** (SEQ ID 2250)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQ GLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYG MDVWGQGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAA*G****
(SEQ ID 2251)

*MDMRVPAQLLGLLLLWLRGARC*DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPK LLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKGSGSGSY VLTQPPSVSVSPGQTASITCSGDKVGHKYASWYQQKPGQSPVLVIYEDSQRPSGIPVRFSGSNSGNT ATLTISGTQAMDEADYYCQAWDSSTDVVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTV EKTVAPTECS* (SEQ ID 2252)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC*QVQLVQSGAEVKKPGSSVKVSCKASGYTFTYRYLHWVRQAPGQ
GLEWMGRIIPVLKITNYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCAVVDDAFDIWGQGTM
VTVSSGSGSGQVQLQQWGAGLLKPSETLSLTCAVSGASFSGHYWTWIRQPPGKGLEWIGEIDHTGS
TNYEPSLRSRVTISVDTSKNQFSLNLKSVTAADTAVYYCARGGQGGYDWGHYHGLDVWGQGTTVTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDT
LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG
KEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESN
GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*
(SEQID 2253)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQ**
GLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCASSKEKATYYYG
MDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPGK* (SEQ ID NO:2254)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK**
GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG
QGTLVTVSSGGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ**
APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLG
SGSG**DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEKRLELVAAINLNGGSTYYS**
DTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYWGQGTLVTVSAGSAASGS
SGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHESPRLLIKYTSQSISGIPS**
RFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGSGSGHHHHHH* (SEQ ID
NO:225)

5

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK**
GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG
QGTLVTVSSGGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ**
APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLG
SGSGGSGSG**DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEKRLELVAAINLNGG**
STYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYWGQGTLVTVSAGS
AASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHESPRLLIKYTSQSI**
SGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGSGSGHHHHHH*
(SEQ ID NO:2256)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK**
GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG
QGTLVTVSSGGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ**
APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVLG
SGSGGSGSGGSSGG**DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEKRLELVAAI**
NLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYWGQGTLVT
VSAGSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSHESPRLLIK**
YTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVKGSGSGHHHH
HH* (SEQ ID NO:2257)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**GS GSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGSYTYYAD SVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS**GGGGSGGGG SGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPERF SGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**HHHHHH* (SEQ ID NO:2258)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**GS GSGGSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGS YTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS**GGGG SGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPS GIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**HHHHHH* (SEQ ID NO:2259)

*MDMRVPAQLLGLLLLWLRGARC***DVKLVESGGGLVKLGGSLRLSCAASGFSFSTSYMSWVRQTPEK RLELVAAINLNGGSTYYSDTVKGRFTISRDNAKNTLYLQMSSLKSEDTAFYYCASELAGYGTPFAYW GQGTLVTVSA**GSAASGSSGGSSSGA**DIVLTQSPATLSVTPGDSVSLSCKASQSISYNLHWYQQKSH ESPRLLIKYTSQSISGIPSRFSGSGSGTDFTLTINNVETEDFGMYFCQQSNSWPLTFGAGTKLEVK**GS GSGGSGSGGSSGG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVI SGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVS S**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYD KSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**HHH HHH* (SEQ ID NO:2260)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEISPYSGSTYYAD SVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWGQGTLVTVSS**GS AASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASF LYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIK**GSGSG**HHHHHH* (SEQ ID NO:2261)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSGGSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEISPYSG STYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWGQGTLV TVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLL IYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIK**GSGSG**HHH HHH* (SEQ ID NO:2262)

FIG. 19 (contd.)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSGGSGSGGSSGG**EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVAEI SPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRGSVMDYWG QGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPG KAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGTKVEIK**GS GSG**HHHHHH* (SEQ ID NO:2263)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGD GSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS**GG GGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNR PSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**HHHHHH* (SEQ ID NO:2264)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSGGSGSG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVS VISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTV SS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQAPVLVIY DKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**GSGSG**HH HHHH* (SEQ ID NO:2265)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSGGSGSGGSSGG**EVQLVESGGGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGK GLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWG QGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWYQQKPGQ APVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYANTLSLVFGGGTKLTVL**G SGSG**HHHHHH* (SEQ ID NO:2266)

*MDMRVPAQLLGLLLLWLRGARC***EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGK GLEWVAEISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALRARPPIRLHPRG SVMDYWGQGTLVTVSS**GSAASGSSGGSSSGA**DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVA WYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQG TKVEIK**GSGSG**DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQP REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**GSGSGEVQLVESGGGLVQPGGSLRLSCA ASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGSYTYYADSVKGRFTISSDNSKNTLYLQMNSLRAE DTAVYYCARNFIKYVFANWGQGTLVTVSS**GGGGSGGGGSGGGGS**DIELTQPPSVSVAPGQTARIS CSGDNIGSFYVHWYQQKPGQAPVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQ SYANTLSLVFGGGTKLTVL* (SEQ ID NO:2267)**

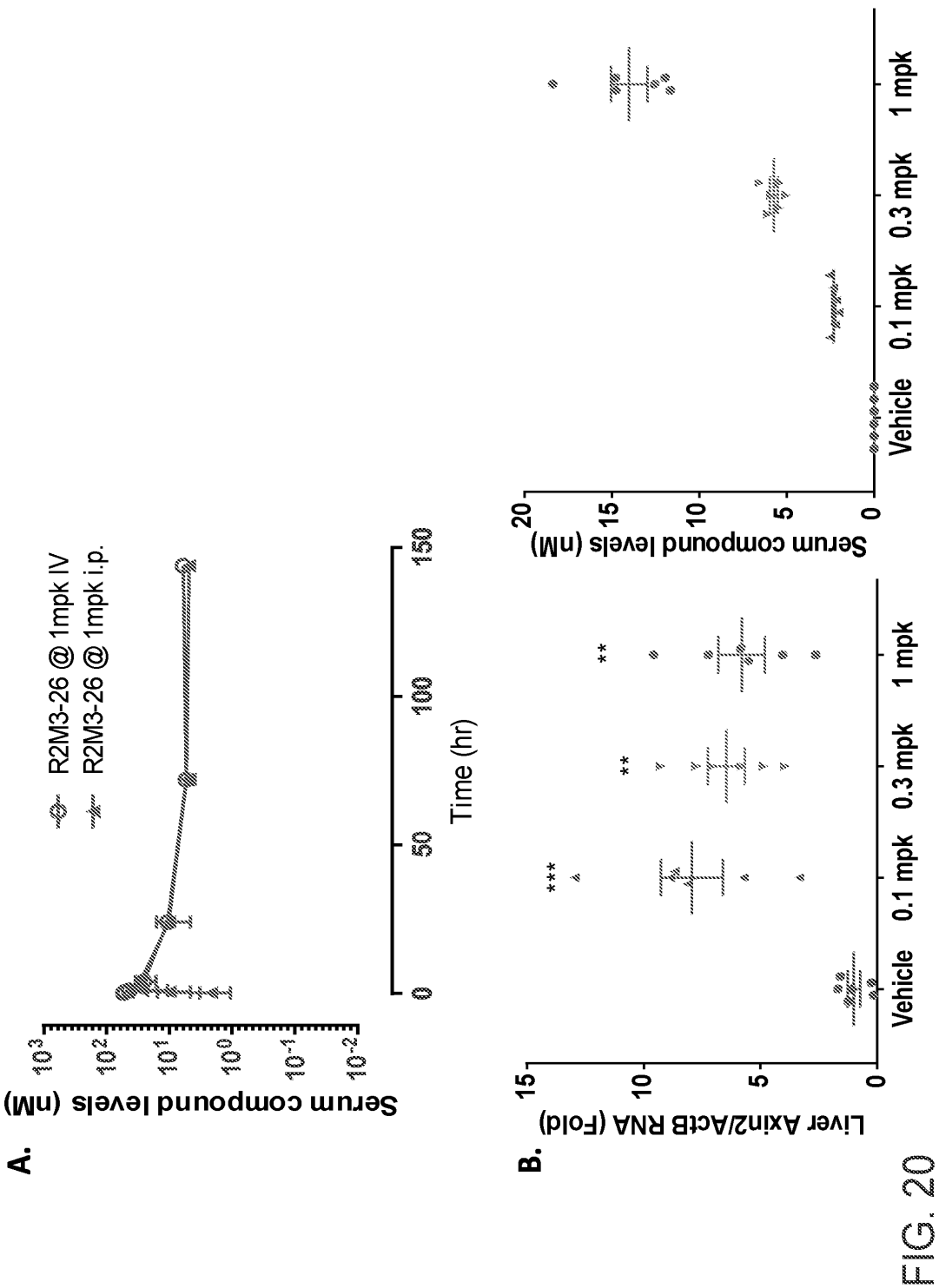

A.
R2M3-26 @ 1mpk IV
R2M3-26 @ 1mpk i.p.
Serum compound levels (nM)
Time (hr)
B.
Liver Axin2/ActB RNA (Fold)
Serum compound levels (nM)
Vehicle
0.1 mpk
0.3 mpk
1 mpk

FIG. 20

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	SRZN-006/04US 328202-2203
	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
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	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

City	Burlingame	State/Province	CA	Country of Residence	US

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	SRZN-006/04US 328202-2203
	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Mailing Address of Inventor:

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	SRZN-006/04US 328202-2203
	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Mailing Address of Inventor:

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Postal Code	94080	**Country**	US

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	SRZN-006/04US 328202-2203
	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Application Information:

Title of the Invention	WNT SURROGATE MOLECULES AND USES THEREOF		
Attorney Docket Number	SRZN-006/04US 328202-2203	**Small Entity Status Claimed** ☐	
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	132	**Suggested Figure for Publication (if any)**	

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For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

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Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

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This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	16/954484	2020-06-16
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
16/954484	a 371 of international	PCT/US2018/066616	2018-12-19
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2018/066616	Claims benefit of provisional	62/607875	2017-12-19
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2018/066616	Claims benefit of provisional	62/641217	2018-03-09
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2018/066616	Claims benefit of provisional	62/680522	2018-06-04
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	SRZN-006/04US 328202-2203
	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

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Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

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B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

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☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

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	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◯ Assignee	◯ Legal Representative under 35 U.S.C. 117	◯ Joint Inventor
◉ Person to whom the inventor is obligated to assign.	◯ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	Surrozen Operating, Inc.		
Mailing Address Information For Applicant:			
Address 1	171 Oyster Point Blvd., Suite 400		
Address 2			
City	South San Francisco	**State/Province**	CA
Country	US	Postal Code	94080
Phone Number		Fax Number	
Email Address			

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Assignee Information including Non-Applicant Assignee Information:

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	Application Number	
Title of Invention	WNT SURROGATE MOLECULES AND USES THEREOF	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

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Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

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This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Carol D. Laherty/			Date (YYYY-MM-DD)	2023-07-28
First Name	Carol D.	Last Name	Laherty	Registration Number	51,909

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	Application Number	
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Application Number	Not yet assigned
Filing Date	Filed concurrently herewith
First Named Inventor	Yang LI
Title	WNT SURROGATE MOLECULES AND USES THEREOF
Art Unit	Not yet assigned
Examiner Name	Not yet assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

SIGNATURE of Applicant or Patent Practitioner

Signature	/Carol D. Laherty/	Date (Optional)	
Name	Carol D. Laherty	Registration Number	51,909
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

[X] *Total of 1 forms are submitted.

Doc Code: PA..
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 157070

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

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[X] The address associated with the above-mentioned Customer Number

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I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Surrozen Operating, Inc.

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

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SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	Nov. 12, 2021
Name	Wen-Chen Yeh		
Title	Chief Scientific Officer		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

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259663625

WNT SURROGATE MOLECULES AND USES THEREOF

Cross Reference to Related Applications

This application is a continuation of U.S. Application No. 16/954,484, which is a National Stage Application under 35 U.S.C. § 371 of International Application No. PCT/US2018/066616, filed December 19, 2018, which claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/607,875, filed December 19, 2017, US Provisional Application No. 62/641,217, filed March 9, 2018, and U.S. Provisional Application No. 62/680,522, filed June 4, 2018, each of which is incorporated by reference herein in its entirety.

Statement Regarding Sequence Listing

The Sequence Listing XML associated with this application is provided in XML file format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing XML is SRZN_006_04US_ST26.xml. The XML file is 2,969,063 bytes, and created on July 28, 2023, and is being submitted electronically via USPTO Patent Center.

BACKGROUND

Technical Field

The present invention relates generally to Wnt signaling pathway agonist molecules, compositions, and methods of using the same. Such molecules are useful, for example, in modulating Wnt signaling pathways.

Description of the Related Art

Wnt ("Wingless-related integration site" or "Wingless and Int-1" or "Wingless-Int") ligands and their signals play key roles in the control of development, homeostasis and regeneration of many essential organs and tissues, including bone, liver, skin, stomach, intestine, kidney, central nervous system, mammary gland, taste bud, ovary, cochlea and many other tissues

(reviewed, e.g., by Clevers, Loh, and Nusse, 2014; 346:1248012). Modulation of Wnt signaling pathways has potential for treatment of degenerative diseases and tissue injuries.

One of the challenges for modulating Wnt signaling as a therapeutic is the existence of multiple Wnt ligands and Wnt receptors, Frizzled 1-10 (Fzd1-10), with many tissues expressing multiple and overlapping Fzds. Canonical Wnt signals also involve Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) or Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) as co-receptors, which are broadly expressed in various tissues, in addition to Fzds. Accordingly, there is clearly a need in the art for binding moieties that specifically bind to one or more Fzd, LRP5, or LRP6 to modulate Wnt signaling pathways. The present invention addresses this need.

BRIEF SUMMARY

In various embodiments, the present invention provides WNT surrogate molecules and related uses thereof.

In one embodiment, the disclosure provides a soluble, bivalent, bispecific Wnt surrogate molecule, wherein the Wnt surrogate molecule comprises: (i) one or more regions that specifically binds to one or more Frizzled (Fzd) receptor (a Fzd binding region); and (ii) one or more regions that specifically binds to a Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) and/or a Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (a LRP5/6 binding region).

In particular embodiments, the Wnt surrogate molecule comprises two or more Fzd binding regions and two or more LRP5/6 binding regions. In particular embodiments, one or more Fzd binding regions comprise one or more antigen-binding fragments of an antibody. In particular embodiments, one or more antigen-binding fragments are selected from the group consisting of: IgG, scFv, Fab, and VHH or sdAb

In particular embodiments, any of the Fzd antigen-binding fragments comprise: (i) CDRH1, CDRH2 and CDRH3 sequences set forth for any of the antibodies of Tables 1A or 1B; and/or (ii) CDRL1, CDRL2 and CDRL3 sequences set forth for any of the antibodies of Tables 1A or 1B, or a

variant of said Fzd binding region comprising one or more amino acid modifications, wherein said variant comprises less than 8 amino acid substitutions in said CDR sequences. In particular embodiments, any of the Fzd binding regions comprise an amino acid sequence having at least 90% identity to any of the sequences set forth in SEQ ID NOs:1-65 or 129-132, or an antigen-binding fragment thereof.

In particular embodiments, any of the Fzd binding regions bind to one or more of Frizzled 1 (Fzd1), Frizzled 2 (Fzd2), Frizzled 3 (Fzd3), Frizzled 4 (Fzd4), Frizzled 5 (Fzd5), Frizzled 6 (Fzd6), Frizzled 7 (Fzd7), Frizzle 8 (Fzd8), Frizzled 9 (Fzd9), and Frizzled 10 (Fzd10). In particular embodiments, any of the Fzd binding region binds to two or more of Frizzled 1 (Fzd1), Frizzled 2 (Fzd2), Frizzled 3 (Fzd3), Frizzled 4 (Fzd4), Frizzled 5 (Fzd5), Frizzled 6 (Fzd6), Frizzled 7 (Fzd7), Frizzled 8 (Fzd8), Frizzled 9 (Fzd9), and Frizzled 10 (Fzd10). In particular embodiments, any of the Fzd binding region binds to: (i) Fzd1, Fzd2, Fzd7 and Fzd9; (ii) Fzd1, Fzd2 and Fzd7; (iii) Fzd5 and Fzd8; (iv) Fzd5, Fzd7 and Fzd8; (v) Fzd1, Fzd4, Fzd5 and Fzd8; (vi) Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8; (vii) Fzd4 and Fzd9; (viii) Fzd9 and Fzd10; (ix) Fzd5, Fzd8 and Fzd10; or (x) Fzd4, Fzd5 and Fzd8; Fzd1, Fzd5, Fzd7 and Fzd8.

In particular embodiments, any of the surrogate molecules comprise one or more LRP5/6 binding regions comprise one or more antigen-binding fragments of an antibody. In particular embodiments, the one or more antigen-binding fragments are selected from the group consisting of: IgG, scFv, Fab, and VHH or sdAb In particular embodiments, any of the one or more LRP5/6 binding regions or antigen-binding fragments comprise: (i) CDRH1, CDRH2 and CDRH3 sequences set forth for any of the antibodies of Table 2; and/or (ii) CDRL1, CDRL2 and CDRL3 sequences set forth for any of the antibodies of Table 2, or a variant of said LRP5/6 binding region comprising one or more amino acid modifications, wherein said variant comprises less than 8 amino acid substitutions in said CDR sequences. In particular embodiments, any of the one or more LRP5/6 binding regions comprise an amino acid sequence having at least 90% identity to any of the sequences set forth in SEQ ID NOs:66-88 or 133, or an antigen-binding fragment thereof.

In particular embodiments of any of the Wnt surrogate molecules, the Fzd binding region comprising a Fab, and the LRP5/6 binding region comprising a VHH or sdAb In particular embodiments, the Fab is present within a full immunoglobulin (Ig), optionally an IgG, comprising a light chain and a heavy chain. In particular embodiments, the LRP5/6 binding region is fused to the N-terminus or the C-terminus of the heavy chain. In particular embodiments, the LRP5/6 binding region is fused to the N-terminus or the C-terminus of the light chain. In certain embodiments, the LRP5/6 binding region is fused to the N-terminus of the heavy chain of the full Ig or the N-terminus of the light chain of the full Ig. In certain embodiments, the LRP5/6 binding region is fused to the C-terminus of the heavy chain of the full Ig or the C-terminus of the light chain of the full Ig. In certain embodiments, the variable light chain region of the LRP5/6 binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig. In certain embodiments, the variable light chain region of the LRP5/6 binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the LRP5/6 binding Fab is fused to the N-terminus of the variable light chain region of the full IgG. In particular embodiments, any of the LRP5/6 binding region is fused to the heavy chain or the light chain via one or more linker moiety.

In certain embodiments of any of the Wnt surrogate molecules, the Fzd binding region comprises a VHH or sdAb and the LRP5/6 binding region comprises a Fab. In particular embodiments, the Fab is present within a full immunoglobulin (Ig), optionally an IgG, comprising a light chain and a heavy chain. In certain embodiments, the Fzd binding region is fused to the N-terminus or the C-terminus of the heavy chain. In certain embodiments, the Fzd binding region is fused to the N-terminus or the C-terminus of the light chain. In some embodiment, the Fzd binding region is fused to the N-terminus of the heavy chain of the full Ig or the N-terminus of the light chain of the full Ig. In some embodiments, the Fzd binding region is fused to the C-terminus of the heavy chain of the full Ig or the C-terminus of the light chain of the full Ig.In some embodiments, the variable light chain region of the Fzd binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig. In some

embodiments, the variable light chain region of the Fzd binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the Fzd binding Fab is fused to the N-terminus of the variable light chain region of the full IgG. In certain embodiments, any of the the Fzd binding region is fused to the heavy chain or the light chain via one or more linker moiety.

In another embodiment, any of the Fzd binding region comprises a Fab or Fv, and the LRP5/6 binding region comprises a Fab or Fv. In particular embodiments, the Fab of the Fzd binding region or the Fab or Fv of the LRP5/6 binding region is present within a full immunoglobulin (Ig), optionally an IgG, comprising a light chain and a heavy chain. In particular embodiments, only one of the Fab of the Fzd binding region or the Fab of the LRPp5/6 binding region is present within the full immunoglobulin (Ig). In particular embodiments, the Fab or Fv of the Fzd binding region is present within the full Ig. In particular embodiments, the Fab or Fv of the LRP5/6 binding region is fused to the N-terminus of the Ig. In particular embodiments, the Fab of the LRP5/6 binding region is fused to the C-terminus of the Ig. In a further embodiment, the Fab of the LRP5/6 binding region is present within the full Ig. In particular embodiments, the Fab of the Fzd binding region is fused to the N-terminus of the Ig. In particular embodiments, the Fab of the Fzd binding region is fused to the C-terminus of the Ig. In some embodiments, the variable light chain region of the Fzd binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the Fzd binding Fab is fused to the N-terminus of the variable light chain region of the full IgG. In some embodiments, the variable light chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable light chain region of the full IgG. In some embodiments, the variable heavy chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable light chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable light chain region of the full IgG. In some embodiments, variable light chain region of the Fzd binding Fv is fused to the N-terminus of the variable heavy chain

region of the full Ig, and the variable heavy chain region of the Fzd binding Fv is fused to the N-terminus of the variable light chain region of the full IgG. In some embodiments, the variable heavy chain region of the Fzd binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable light chain region of the Fzd binding Fv is fused to the N-terminus of the variable light chain region of the full IgG.

In another embodiment, any of the Fzd binding regions comprises a VHH or sdAb, and the LRP5/6 binding region comprises a VHH or sdAb. In particular embodiments, the Fzd binding region is fused to the Lrp5/6 binding region, and wherein the Fzd binding region or the LRP5/6 binding region is fused to an Fc region. In particular embodiments, the Fzd binding region is fused to the N-terminus of an Fc region, and wherein the LRP5/6 binding region is fused to the C-terminus of an Fc region. In particular embodiments, the Fzd binding region is fused to the C-terminus of an Fc region, and wherein the LRP5/6 binding region is fused to the N-terminus of an Fc region.

In a further embodiment, any of the antibodies or one or more antigen-binding fragment thereof, is humanized. In a further embodiment, any of the Wnt surrogate molecules binds to one or more Fzd receptor with a K_D of 50 μM or lower. In further embodiments, any of the Wnt surrogate molecules binds to LRP5 and/or LRP6 with a K_D of 50 μM or lower.

In a further embodiment, any of the Wnt surrogate molecule modulates a Wnt signaling pathway in a cell, optionally a mammalian cell. In particular embodiments, the Wnt surrogate molecule increases signaling via the Wnt signaling pathway in the cell. In particular embodiments, the Wnt signaling pathway is a canonical Wnt signaling pathway or a non-canonical Wnt signaling pathway.

In a related embodiment, the present disclosure provides an isolated polynucleotide encoding a polypeptide sequence comprising one or more of the Fzd binding regions and/or one or more of the LRP5/6 binding regions of a Wnt surrogate molecule. In particular embodiments, the present disclosure provides an expression vector comprising the isolated polynucleotide. In further particular embodiments, the present disclosure provides an isolated host cell comprising the expression vector.

In a related embodiment, the present disclosure provides a pharmaceutical composition comprising a physiologically acceptable excipient, diluent, or carrier, and a therapeutically effective amount of any of the Wnt surrogate molecules disclosed herein.

In related embodiments, the present disclosure provides a method for agonizing a Wnt signaling pathway in a cell, comprising contacting the cell any of the Wnt surrogate molecules, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

In particular embodiments, the present disclosure provides a method for treating a subject having a disease or disorder associated with reduced Wnt signaling, comprising administering to the subject an effective amount of the pharmaceutical composition, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway. In particular embodiments, the disease or disorder is selected from the group consisting of: bone fractures, osteoporosis (e.g., post-menopausal osteoporosis), osteoporotic fractures, spinal fusion, vertebral compression fracture, pre-operative spinal surgery optimization, osseointegration of orthopedic devices, tendon-bone integration, tooth growth and regeneration, dental implantation, periodontal diseases, maxillofacial reconstruction, osteonecrosis of the jaw, osteoarthritis (OA), muscular dystrophy, muscle atrophy resulting from sarcopenia or cachexia, alopecia, hearing loss, including regeneration of inner and outer auditory hair cells, vestibular hypofunction, macular degeneration, vitreoretinopathy, diseases of retinal degeneration, including diabetic retinopathy, diseases/disorders affecting the integrity of the blood brain barrier, Fuchs' dystrophy, stroke, traumatic brain injury, Alzheimer's disease, multiple sclerosis, spinal cord injuries, oral mucositis, short bowel syndrome, inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), in particular IBD with fistula formation, metabolic syndrome, diabetes, dyslipidemia, pancreatitis, exocrine pancreatic insufficiency, wound healing, diabetic foot ulcers, coronary artery disease, acute kidney injuries, chronic kidney diseases, chronic obstructive pulmonary diseases (COPD), pulmonary fibrosis, including idiopathic pulmonary fibrosis, acute liver failure, acute alcoholic liver injuries, chronic liver diseases with

hepatitis C virus (HCV), HCV subjects post-antiviral drug therapies, chronic liver diseases with hepatitis B virus (HBV), fibrosis, HBV subjects post-antiviral drug therapies, chronic alcoholic liver diseases, alcoholic hepatitis, non-alcoholic fatty liver diseases and non-alcoholic steatohepatitis (NASH), cirrhosis, and chronic liver insufficiencies of all causes. In certain embodiments, the disease or disorder is a bone disease or disorder. In particular embodiments, the disease or disorder is a bone disease or disorder, and the Wnt surrogate molecule binds Fzd1, Fzd2, and FZD7, and binds LRP5 and/or LRP6. In certain embodiments, the disease or disorder is a bone disease or disorder, and the Wnt surrogate molecule binds Fzd1, Fzd2, FZD7, Fzd5 and Fzd8, and also binds LRP5 and/or LRP6.

In another related embodiment, the present disclosure provides a method for increasing bone mineral density, increasing bone volume, increasing bone cortical thickness, increasing bone mineral apposition rate, increasing bone stiffness, increasing bone biomechanical strength, increasing resistance to bone fracture, decreasing bone resorption, or decreasing bone loss associated with osteoporosis, in a subject in need thereof, comprising providing to the subject an effective amount of a pharmaceutical composition comprising a Wnt surrogate molecule, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway. In certain embodiments, the Wnt surrogate molecule binds Fzd1, Fzd2, and FZD7, and binds LRP5 and/or LRP6. In certain embodiments, the Wnt surrogate molecule binds Fzd1, Fzd2, FZD7, Fzd5 and Fzd8, and also binds LRP5 and/or LRP6.

In particular embodiments, methods of the invention, including those related to treating or preventing a bone disease or disorder, such as osteoporosis (e.g., post-menopausal osteoporosis), further comprise providing the subject an antiresorptive agent (in combination with a Wnt surrogate molecule). Examples of anti-resorptive agents include, but are not limited to, bisphosphonates or selective estrogen receptor modulators. Antiresorptive agents are used to increase bone strength in individuals with osteoporosis and include five principal classes of agents: bisphosphonates, estrogens, selective estrogen receptor modulators (SERMs), calcitonin and monoclonal antibodies such as denosumab, any of which may be used. Illustrative examples of

antiresorptive agents include, but are not limited to: bisphosphonates, e.g., alendronate -generic medication (Brand name: Fosamax™, Fosamax™ Plus D), risedronate (Brand name: Actonel™, Actonel™ with Calcium), ibandronate (Brand name: Boniva™), and zoledronic acid (Brand name: Reclast™); other antiresorptives, e.g., estrogen therapy or hormone therapy, raloxifene (Brand name: Evista™), and denosumab (Prolial™); and anabolic medication, e.g., teriparatide (Forteo™).

In a further related embodiment, the present disclosure provides a method for increasing liver to body weight ratio, promoting liver regeneration, increasing liver cell proliferation or mitosis, decreasing liver fibrosis, optionally following a chronic liver injury, increasing hepatocyte function, or decreasing coagulation time in liver, in a subject in need thereof, comprising providing to the subject an effective amount of a pharmaceutical composition comprising a Wnt surrogate molecule, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-D. Schematic diagrams of illustrative formats of Wnt surrogate molecules.

FIGS. 2A-2D. Characterization of a Wnt surrogate molecule, R2M3-26.

FIGS. 3A-3D. Characterization of a Wnt surrogate molecule,, R2M3-32.

FIGS. 4A-4B. Graphs showing that R2M3-26 and R2M3-32 activities can be inhibited by soluble Fzd ECD and by R2M3 IgG alone without the Lrp binding arm.

FIG. 5. Characterization of illustrative R2M3-Lrp6 binder fusions in 293, Huh7, A375, and BNL.CL2 Wnt dependent reporter assays.

FIG. 6. Characterization of illustrative 18R5-Lrp6 binder fusions in 293, A375, and BNL.CL2 Wnt dependent reporter assays.

FIG. 7. Characterization of illustrative18R5-Lrp5 binder fusions in 293 Wnt dependent reporter assays.

FIG. 8A-8B. Characterization of illustrative Fzd binders-Lrp6 binder 26 fusions in 293 Wnt dependent reporter assays.

FIG. 9. SAR analysis of illustrative Wnt surrogate molecules in the IgG-Nab fusion format.

FIG. 10A-10B. Characterization of R2M3-26 in the Fab format in 293 Wnt dependent reporter assays.

FIG. 11A-11B. Characterization of R2M3-32 in the Fab format in 293 Wnt dependent reporter assays.

FIG. 12A-12B. Characterization of R2M3-26 in the Hetero-Ig format in 293 Wnt dependent reporter assays.

FIG. 13. Characterization of 26-17SB9 in the VHH/sdAb-VHH/sdAb format, in different tandem formats, and on different ends of the Fc fragment in 293 Wnt dependent reporter assays.

FIG. 14A-14H. Characterization of 18R5-LRP6 Binder Fusions in tandem scFv formats in 293 Wnt dependent reporter assays.

FIG. 15A-15G. Characterization of various Wnt Surrogate molecules in the Fab-IgG format in 293 Wnt dependent reporter assays.

FIG. 16A-16C. Characterization of R2M3-26 in the F(ab')2 format in 293 Wnt dependent reporter assays.

FIG. 17A-17H. Characterization of additional Wnt surrogate molecules in 293 Wnt dependent reporter assays

FIG. 18A-18C. A. Schematic diagram of the 2Fv-Ig format. B-C. Characterization of the Wnt surrogate molecule, 10SG11-1RC07.

FIG. 19. Sequences of polypeptide chains of illustrative Wnt surrogates molecules.

FIG. 20A-20B. In vivo PK/PD characterization of R2M3-26

FIGS. 21A-21E. Images and graphs showing that systemic expression of 18R5-DKK1c for 14 days results in increased bone mineral density. *P value <0.05; ** P value <0.0001. For each time point, the bars from left to right are as follows: vehicle (diamond), romosozumab (square), AAV CAG-GFP (triangle), AAV ScFv (anti-GFP)-DKK1cF234K-Flag-His (inverted triangle), and AAV 18R5-DKK1c-FLagHis (circle).

FIGS. 22A-22D. Images and graphs showing that systemic expression of 18R5-DKK1c for 14 days or 28 days results in increased bone volume. For each time point, the bars from left to right are as follows: vehicle, romosozumab, AAV CAG-GFP, AAV ScFv (anti-GFP)-DKK1cF234K-Flag-His, and AAV 18R5-DKK1c-FLagHis. *P value <0.05; ** P value <0.0001, **** P value <0.0001.

FIGS.23A-23B. Graphs showing the dynamic parameters of bone formation based on fluorochrome labelling. For each time point, the bars from left to right are as follows: vehicle, romosozumab, AAV CAG-GFP, AAV ScFv (anti-GFP)-DKK1cF234K-Flag-His, and AAV 18R5-DKK1c-FlagHis.

FIGS. 24A-24D. Graphs and images showing that systemic expression of 18R5-DKK1c results in increased osteoblast and reduced osteoclast on bone surface. For each time point, the bars from left to right are as follows: vehicle, romosozumab, AAV CAG-GFP, AAV ScFv (anti-GFP)-DKK1cF234K-Flag-His, and AAV 18R5-DKK1c-FlagHis. ** P value <0.05.

FIGS. 25A-25C. Diagram of assay for bone stiffness and fracture and graphs showing ultimate load to failure and stiffness in mice treated as indicated.

FIGS. 26A-26D. Graphs and images showing that systemic treatment with R2M3-26 results in rapid and sustained increase in bone after one week. For each timepoint, the bars from left to right correspond to the treatments indicated from top to bottom. **** indicates P value <0.0001.

FIGS. 27A-27C. Images and graphs showing that R2M3-26 treatment rapidly reverses the bone loss associated with ovariectomy-induced

osteoporosis. For each time point, the bars from left to right correspond to the treatments indicated from top to bottom.

FIGS. 28A-28C. Images and graphs showing that a single injection of R2M3-26 rapidly increases bone volume.

FIGS. 29A-29D. Graphs showing that high doses of R2M3-26 and 1R-C07-26 significantly and rapidly increase bone formation in naïve mice. For each timepoint, the bars from left to right correspond to the treatments indicated from top to bottom.

FIG. 30. Graphs showing that R2M3-26 and 1R-C07-3 increase bone mineral density in naïve mice. For each timepoint, the bars from left to right correspond to the treatments indicated from top to bottom.

FIG. 31. Graph showing changes in whole body bone mineral density (BMD) measured weekly in ovariectomized mice as compared with naïve and sham surgery operated mice.

FIG 32. Changes in vertebral mineral density (image shown) and changes the vertebral resistance to compression fracture in vertebra isolated from mice after various treatments (bar graph) as measured in newton units of force (N) after 4 weeks of treatment.

FIGS. 33A-D. Test of Wnt surrogate molecules in an Einhorn fracture model. Radiographs of the callus after 1 week (A) and 6 weeks (B) of treatment with Wnt surrogate molecules are shown. Graphs of changes in whole body bone mineral density (BMD) in contralateral femur are shown (C). Scatter plots showing changes in callus tissue volume, callus bone volume, bone volume/tissue volume ratio (BV/TV), and bone mineral content per millimeter (BMC/mm) as shown in (D) along with representative images of bone slices are shown.

FIG. 34. Graph with changes in whole body bone mineral density (BMD) measured weekly with different Wnt surrogate molecule dosing schedules is shown.

FIG 35. Graph with changes in whole body bone mineral density (BMD) measured weekly from mice treated with different Wnt surrogate molecule alone and in combination with Romosozumab is shown.

FIG 36. Levels of therapeutic molecules in serum as measured by ELISA. These data accompany gene expression data presented in Table 4.

FIGS. 37A-37C. Liver (A), small intestine (B) and colon (C) to body weight ratio after treatment with AAV-delivered Wnt surrogates. (**) $p<0.01$. For each graph, the treatments shown from left to right correspond to those in the legend from top to bottom.

FIGS. 38A-38B. Body weight (A) and liver to body weight (B) ratio after treatment with recombinantly produced Wnt surrogate proteins. (*) $p<0.05$. For each time point, the treatments shown from left to right correspond to those in the legend from top to bottom.

FIGS. 39A-39D. Induction of proliferation markers in response to R2M3-26 and Rspo2 recombinant proteins. Liver Ki67 (A) and cyclinD1 (B) mRNA expression. Average count of PCNA (C) or phospho-histone H3 (D) positive nuclei per 10x field after immunohistochemistry staining with PCNA and phospho-histone H3 antibodies respectively. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (****) $p<0.0001$. For each time point, the treatments shown from left to right correspond to those in the legend from top to bottom.

FIGS. 40A-40H. Efficacy of AAV-delivered Wnt surrogate and R-Spondin in a thioacetamide-induced chronic liver disease model. Design for study 1 (A) and study 2 (B). Liver to body weight ratio (C-D), liver weight (E-F), liver collagen A1 mRNA expression (G) and percentage red area in liver histological sections stained with Sirius red (H) in response to AAV-delivered wnt surrogate and R-spondin in study 1 (C, E, G and H) and study 2 (D, F, H). (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$, (****) $p<0.0001$. For each graph, the treatments shown from left to right correspond to those in the legend from top to bottom (not including baseline).

FIGS. 41A-41N. Efficacy of recombinantly produced Wnt surrogate and R-Spondin in a thioacetamide-induced chronic liver disease

model. Study design (A). D-2, D0, D3, D7, D10, D14 represents days relative to the start of treatment with recombinant proteins. Liver to body weight ratio (B, C), liver axin2 mRNA (D, E), cyclinD1 mRNA (F, G) and Ki67 mRNA (H, I) expression, average count of PCNA (J, K) or phospho-histone H3 (L, M) positive nuclei per 10x field after immunohistochemistry staining with PCNA and phospho-histone H3 antibodies respectively, in a study using Rspo2 mono treatment (B, D, F, H, J, L) or R2M3-26/Rspo2 combination treatment (C, E, G, I, K, M). Pro-thrombin time ratio relative to the average pro-thrombin time in plasma collected from control naïve mice without TAA exposure (N). (∗) $p<0.05$, (∗∗) $p<0.01$, (∗∗∗∗) $p<0.0001$. No TAA treatment is indicated by the dashed line. For each bar graph timepoint, the treatments shown from left to right correspond to those in the legend shown above from top to bottom.

FIGS. 42A-42C. Efficacy of recombinantly produced Wnt surrogate and R-Spondin in a CCl4-induced chronic liver disease model. Study design (A). Liver to body weight ratio (B) pro-thrombin time (C) and sirius red staining (D) in response to CCl4 treatment, R2M3-26 and Rspo2. (∗) $p<0.05$, (∗∗) $p<0.01$, (∗∗∗) $p<0.001$,(∗∗∗∗) $p<0.0001$. For each graph, the treatments shown from left to right correspond to those in the legend from top to bottom (not including baseline).

FIGS. 43A-43D. Induction of proliferation markers in response to recombinantly produced Wnt surrogate in an acetaminophen-induced acute liver injury model. Study design (A). Serum level of alanine transferase at 24 and 48 hours after treatment with acetaminophen (B). Relative cyclinD1 (C) and Ki67 (D) mRNA expression. (∗) $p<0.05$, (∗∗∗) $p<0.001$, (∗∗∗∗) $p<0.0001$.

FIGS. 44A-44D. Induction of proliferation markers in response to R-Spondin in an acetaminophen-induced acute liver injury model. Study design (A). Serum level of alanine transferase at 24 and 48 hours after treatment with acetaminophen (B). Relative cyclinD1 (C) and Ki67 (D) mRNA expression. (∗∗) $p<0.01$, (∗∗∗) $p<0.001$, (∗∗∗∗) $p<0.0001$.

FIGS. 45A-45D. Induction of proliferation markers in response to Wnt surrogate and R-Spondin in an acetaminophen-induced acute liver injury

model. Study design (A). Serum level of alanine transferase at 24, 36, 48 and 60 hours after treatment with acetaminophen (B). Relative cyclinD1 (C) and Ki67 (D) mRNA expression. (*) $p<0.05$, (****) $p<0.0001$. For each time point, the treatments shown from left to right correspond to those in the legend from top to bottom.

FIGS. 46A-46D. Efficacy of recombinantly produced Wnt surrogate and R-Spondin on the survival of mice after acetaminophen-induced liver injury. Study design (A). Survival curve of mice treated with the control anti-eGFP control protein or R2M3-26 (B), Rspo2 (C) or a combination of R2M3-26 and Rspo2 (D) recombinant proteins.

DETAILED DESCRIPTION

The present disclosure relates to Wnt surrogate molecules that bind to one or more Fzd receptor and one or more LRP5 or LRP6 receptor and modulate a downstream Wnt signaling pathway. In particular embodiments, the Wnt surrogate molecules activate a Wnt signaling pathway or increase signaling via a Wnt signaling pathway. In particular embodiments, the Wnt surrogate molecules disclosed herein comprise: (i) one or more antibodies or antigen-binding fragments thereof that specifically bind to one or more Fzd receptor, including antibodies or antigen-binding fragments thereof having particular Fzd receptor specificity and/or functional properties; and (ii) one or more antibodies or antigen-binding fragments thereof that specifically bind to LRP5 and/or LRP6. Certain embodiments encompass specific structural formats or arrangements of the Fzd binding region(s) and LRP5/6 binding region(s) of Wnt surrogate molecules advantageous in increasing downstream Wnt pathway signaling and related biological effects.

Embodiments of the invention pertain to the use of Wnt surrogate molecules for the diagnosis, assessment and treatment of diseases and disorders associated with Wnt signaling pathways. In certain embodiments, the subject Wnt surrogate molecules are used to modulate a Wnt signaling pathway in a cell or tissue. In certain embodiments, the subject Wnt surrogate molecules are used in the treatment or prevention of diseases and disorders associated with aberrant or deregulated (e.g., reduced) Wnt signaling, or for which modulating, e.g., increasing, Wnt signaling would provide a therapeutic benefit.

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, *Current Protocols in Molecular Biology* or *Current Protocols in Immunology*, John Wiley

& Sons, New York, N.Y.(2009); Ausubel *et al.*, *Short Protocols in Molecular Biology*, 3rd ed., Wiley & Sons, 1995; Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Maniatis *et al. Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, eds., 1984); *Animal Cell Culture* (R. Freshney, ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984) and other like references.

As used in this specification and the appended claims, the singular forms “a,” “an” and “the” include plural references unless the content clearly dictates otherwise.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Each embodiment in this specification is to be applied *mutatis mutandis* to every other embodiment unless expressly stated otherwise.

Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (*e.g.*, electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. These and related techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. Unless specific definitions are provided, the nomenclature utilized in connection with, and the laboratory procedures and techniques of, molecular biology, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and

commonly used in the art. Standard techniques may be used for recombinant technology, molecular biological, microbiological, chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of subjects.

Embodiments of the present invention relate to antibodies and antigen-binding fragments thereof that bind to one or more Fzd receptor. Sequences of illustrative antibodies, or antigen-binding fragments, or complementarity determining regions (CDRs) thereof, are set forth in SEQ ID NOs:1-65 or 129-132, Tables 1A and 1B, and Table 3. Anti-Fzd antibodies and antigen-binding fragments there that may be used or present in the Wnt surrogate molecules disclosed herein include, but are not limited to, those described in the U.S. provisional application no. 62/607,877, titled Anti-Frizzled Antibodies and Methods of Use, Attorney docket no. SRZN-004/00US, filed on December 19, 2017.

Embodiments of the present invention relate to antibodies and antigen-binding fragments thereof that bind to LRP5 and/or LRP6. Sequences of illustrative antibodies, or antigen-binding fragments, or complementarity determining regions (CDRs) thereof, are set forth in SEQ ID NOs:66-88 or 133, Tables 2A and 2B, and Table 3. Anti-LRP5/6 antibodies and antigen-binding fragments there that may be used or present in the Wnt surrogate molecules disclosed herein include, but are not limited to, those described in the U.S. provisional application no. 62/607,879, titled Anti-LRP5/6 Antibodies and Methods of Use, Attorney docket no. SRZN-005/00US, filed on December 19, 2017.

As is well known in the art, an antibody is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one epitope recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also fragments thereof (such as dAb, Fab, Fab', $F(ab')_2$, Fv), single chain (scFv), VHH or sdAb, synthetic variants thereof, naturally occurring

variants, fusion proteins comprising an antibody or an antigen-binding fragment thereof, humanized antibodies, chimeric antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen-binding site or fragment (epitope recognition site) of the required specificity. "Diabodies" or 2scFV-Ig antibodies, are multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al., Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993) are also a particular form of antibody contemplated herein. Minibodies comprising a scFv joined to a CH3 domain are also included herein (S. Hu et al., Cancer Res., 56, 3055-3061, 1996). See *e.g.*, Ward, E. S. *et al.*, Nature 341, 544-546 (1989); Bird et al., Science, 242, 423-426, 1988; Huston et al., PNAS USA, 85, 5879-5883, 1988); PCT/US92/09965; WO94/13804; P. Holliger et al., Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993; Y. Reiter et al., Nature Biotech, 14, 1239-1245, 1996; S. Hu et al., Cancer Res., 56, 3055-3061, 1996.

The term "antigen-binding fragment" as used herein refers to a polypeptide fragment that contains at least one CDR of an immunoglobulin heavy and/or light chain, or of a VHH or sdAb, that binds to the antigen of interest, in particular to one or more Fzd receptor or LRP5 or LRP6 receptor. In this regard, an antigen-binding fragment of the herein described antibodies may comprise 1, 2, 3, 4, 5, or all 6 CDRs of a VH and VL sequence set forth herein from antibodies that bind one or more Fzd receptor or LRP5 and/or LRP6. In particular embodiments, an antigen-binding fragment may comprise all three VH CDRs or all three VL CDRs. Similarly, an antigen binding fragment thereof may comprise all three CDRs of a VHH or sdAb. An antigen-binding fragment of a Fzd-specific antibody is capable of binding to a Fzd receptor. An antigen-binding fragment of a LRP5/6-specific antibody is capable of binding to a LRP5 and/or LRP6 receptor. As used herein, the term encompasses not only isolated fragments but also polypeptides comprising an antigen-binding fragment of an antibody disclosed herein, such as, for example, fusion proteins comprising an antigen-binding fragment of an antibody disclosed herein, such as, e.g., a

fusion protein comprising a VHH or sdAb that binds one or more Fzd receptors and a VHH or sdAb that binds LRP5 and/or LRP6.

The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. In certain embodiments, a binding agent (e.g., a Wnt surrogate molecule or binding region thereof) is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. In certain embodiments, a Wnt surrogate molecule or binding region thereof (e.g., an antibody or antigen-binding fragment thereof) is said to specifically bind an antigen when the equilibrium dissociation constant is $\leq 10^{-7}$ or $\leq 10^{-8}$ M. In some embodiments, the equilibrium dissociation constant may be $\leq 10^{-9}$ M or $\leq 10^{-10}$ M.

In certain embodiments, antibodies and antigen-binding fragments thereof as described herein include a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain framework region (FR) set which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V

region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

The structures and locations of immunoglobulin CDRs and variable domains may be determined by reference to Kabat, E. A. et al., Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (immuno.bme.nwu.edu). Alternatively, CDRs may be determined by using "IMGT®, the international ImMunoGeneTics information system® available at www.imgt.org (see, e.g., Lefranc, M.-P. et al. (1999) *Nucleic Acids Res.*, 27:209-212; Ruiz, M. et al. (2000) *Nucleic Acids Res.*, 28:219-221; Lefranc, M.-P. (2001) *Nucleic Acids Res.*, 29:207-209; Lefranc, M.-P. (2003) *Nucleic Acids Res.*, 31:307-310; Lefranc, M.-P. et al. (2004) *In Silico* Biol., 5, 0006 [Epub], 5:45-60 (2005)]; Lefranc, M.-P. et al. (2005) *Nucleic Acids Res.*, 33:D593-597; Lefranc, M.-P. et al. (2009) *Nucleic Acids Res.*, 37:D1006-1012; Lefranc, M.-P. et al. (2015) *Nucleic Acids Res.*, 43:D413-422).

A “monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody"

encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', $F(ab')_2$, Fv), single chain (scFv), VHH or sdAb, variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen-binding fragment (epitope recognition site) of the required specificity and the ability to bind to an epitope, including Wnt surrogate molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (*e.g.*, by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody".

The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the $F(ab')_2$ fragment which comprises both antigen-binding sites. An Fv fragment for use according to certain embodiments of the present invention can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions of an IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent $V_H::V_L$ heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar *et al.* (1972) *Proc. Nat. Acad. Sci. USA 69*:2659-2662; Hochman *et al.* (1976) *Biochem 15*:2706-2710; and Ehrlich *et al.* (1980) *Biochem 19*:4091-4096.

In certain embodiments, single chain Fv or scFV antibodies are contemplated. For example, Kappa bodies (Ill *et al.*, *Prot. Eng*. 10: 949-57 (1997); minibodies (Martin *et al.*, *EMBO J* 13: 5305-9 (1994); diabodies (Holliger *et al.*, *PNAS* 90: 6444-8 (1993); or Janusins (Traunecker *et al.*, *EMBO*

J 10: 3655-59 (1991) and Traunecker *et al., Int. J. Cancer Suppl*. 7: 51-52 (1992), may be prepared using standard molecular biology techniques following the teachings of the present application with regard to selecting antibodies having the desired specificity. In still other embodiments, bispecific or chimeric antibodies may be made that encompass the ligands of the present disclosure. For example, a chimeric antibody may comprise CDRs and framework regions from different antibodies, while bispecific antibodies may be generated that bind specifically to one or more Fzd receptor through one binding domain and to a second molecule through a second binding domain. These antibodies may be produced through recombinant molecular biological techniques or may be physically conjugated together.

A single chain Fv (scFv) polypeptide is a covalently linked V_H::V_L heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston *et al.* (1988) *Proc. Nat. Acad. Sci. USA 85*(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated—but chemically separated—light and heavy polypeptide chains from an antibody V region into an scFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston *et al.*; and U.S. Pat. No. 4,946,778, to Ladner *et al.*

In certain embodiments, an antibody as described herein is in the form of a diabody. Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (*e.g.* by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

A dAb fragment of an antibody consists of a VH domain (Ward, E. S. *et al.*, Nature 341, 544-546 (1989)).

Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. *Current Opinion Biotechnol.* 4, 446-449 (1993)), *e.g.* prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in *E. coli*. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by knobs-into-holes engineering (J. B. B. Ridgeway et al., *Protein Eng.*, 9, 616-621, 1996).

In certain embodiments, the antibodies described herein may be provided in the form of a UniBody®. A UniBody® is an IgG4 antibody with the hinge region removed (see GenMab Utrecht, The Netherlands; see also, *e.g.*, US20090226421). This proprietary antibody technology creates a stable, smaller antibody format with an anticipated longer therapeutic window than current small antibody formats. IgG4 antibodies are considered inert and thus do not interact with the immune system. Fully human IgG4 antibodies may be modified by eliminating the hinge region of the antibody to obtain half-molecule fragments having distinct stability properties relative to the corresponding intact IgG4 (GenMab, Utrecht). Halving the IgG4 molecule leaves only one area on the UniBody® that can bind to cognate antigens (*e.g.*, disease targets) and the UniBody® therefore binds univalently to only one site on target cells.

In certain embodiments, the antibodies of the present disclosure may take the form of a VHH or sdAb. VHH or sdAb technology was originally developed following the discovery and identification that *camelidae* (e.g., camels and llamas) possess fully functional antibodies that consist of heavy chains only and therefore lack light chains. These heavy-chain only antibodies contain a single variable domain(V_{HH}) and two constant domains (C_H2, C_H3). The cloned and isolated single variable domains have full antigen binding capacity and are very stable. These single variable domains, with their unique structural and functional properties, form the basis of "VHH or sdAb". VHH or sdAb are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts *e.g. E. coli* (see *e.g.* U.S. Pat. No. 6,765,087), molds (for example *Aspergillus* or *Trichoderma*) and yeast (for example *Saccharomyces, Kluyvermyces, Hansenula* or *Pichia* (see *e.g.* U.S. Pat. No. 6,838,254). The production process is scalable and multi-kilogram quantities of VHH or sdAb have been produced. VHH or sdAb may be formulated as a ready-to-use solution having a long shelf life. The VHH or sdAb method (see, e.g., WO 06/079372) is a proprietary method for generating VHH or sdAb against a desired target, based on automated high-throughput selection of B-cells. VHH or sdAb are single-domain antigen-binding fragments of camelid-specific heavy-chain only antibodies. VHH antibodies or sdAb, typically have a small size of around 15 kDa.

In certain embodiments, the antibodies or antigen-binding fragments thereof as disclosed herein are humanized. This refers to a chimeric molecule, generally prepared using recombinant techniques, having an antigen-binding site derived from an immunoglobulin from a non-human species and the remaining immunoglobulin structure of the molecule based upon the structure and/or sequence of a human immunoglobulin. The antigen-binding site may comprise either complete variable domains fused onto constant domains or only the CDRs grafted onto appropriate framework regions in the variable domains. Epitope binding sites may be wild type or modified by one or more amino acid substitutions. This eliminates the constant region as an immunogen

in human individuals, but the possibility of an immune response to the foreign variable region remains (LoBuglio, A. F. *et al.*, (1989) *Proc Natl Acad Sci USA* 86:4220-4224; Queen *et al.*, *PNAS* (1988) 86:10029-10033; Riechmann *et al.*, *Nature* (1988) 332:323-327). Illustrative methods for humanization of the anti-Fzd antibodies disclosed herein include the methods described in U.S. patent no. 7,462,697.

Another approach focuses not only on providing human-derived constant regions, but modifying the variable regions as well so as to reshape them as closely as possible to human form. It is known that the variable regions of both heavy and light chains contain three complementarity-determining regions (CDRs) which vary in response to the epitopes in question and determine binding capability, flanked by four framework regions (FRs) which are relatively conserved in a given species and which putatively provide a scaffolding for the CDRs. When nonhuman antibodies are prepared with respect to a particular epitope, the variable regions can be "reshaped" or "humanized" by grafting CDRs derived from nonhuman antibody on the FRs present in the human antibody to be modified. Application of this approach to various antibodies has been reported by Sato, K., *et al.*, (1993) *Cancer Res* 53:851-856. Riechmann, L., *et al.*, (1988) *Nature* 332:323-327; Verhoeyen, M., *et al.*, (1988) *Science* 239:1534-1536; Kettleborough, C. A., *et al.*, (1991) *Protein Engineering* 4:773-3783; Maeda, H., *et al.*, (1991) *Human Antibodies Hybridoma* 2:124-134; Gorman, S. D., *et al.*, (1991) *Proc Natl Acad Sci USA* 88:4181-4185; Tempest, P. R., *et al.*, (1991) *Bio/Technology* 9:266-271; Co, M. S., *et al.*, (1991) *Proc Natl Acad Sci USA* 88:2869-2873; Carter, P., *et al.*, (1992) *Proc Natl Acad Sci USA* 89:4285-4289; and Co, M. S. *et al.*, (1992) *J Immunol* 148:1149-1154. In some embodiments, humanized antibodies preserve all CDR sequences (for example, a humanized mouse antibody which contains all six CDRs from the mouse antibodies). In other embodiments, humanized antibodies have one or more CDRs (one, two, three, four, five, six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs from the original antibody.

In certain embodiments, the antibodies of the present disclosure may be chimeric antibodies. In this regard, a chimeric antibody is comprised of an antigen-binding fragment of an antibody operably linked or otherwise fused to a heterologous Fc portion of a different antibody. In certain embodiments, the heterologous Fc domain is of human origin. In other embodiments, the heterologous Fc domain may be from a different Ig class from the parent antibody, including IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG (including subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. In further embodiments, the heterologous Fc domain may be comprised of CH2 and CH3 domains from one or more of the different Ig classes. As noted above with regard to humanized antibodies, the antigen-binding fragment of a chimeric antibody may comprise only one or more of the CDRs of the antibodies described herein (*e.g.*, 1, 2, 3, 4, 5, or 6 CDRs of the antibodies described herein), or may comprise an entire variable domain (VL, VH or both).

Wnt Surrogates

The disclosure provides, in certain aspects, Wnt surrogate molecules that bind both one or more Fzd receptors and one or both of LRP5 and/or LRP6. Wnt surrogate molecules may also be referred to as "Wnt surrogates" or "Wnt mimetics." In particular embodiments, the Wnt surrogate molecules bind one or more human Fzd receptors and one or both of a human LRP5 and/or a human LRP6.

In certain embodiments, a Wnt surrogate molecule is capable of modulating or modulates Wnt signaling events in a cell contacted with the Wnt surrogate molecule. In certain embodiments, the Wnt surrogate molecule increases Wnt signaling, e.g., via the canonical Wnt/β-catenin pathway. In certain embodiments, the Wnt surrogate molecule specifically modulates the biological activity of a human Wnt signaling pathway.

Wnt surrogate molecules of the present invention are biologically active in binding to one or more Fzd receptor and to one or more of LRP5 and LRP6, and in activation of Wnt signaling, i.e., the Wnt surrogate molecule is a Wnt agonist. The term "Wnt agonist activity" refers to the ability of an agonist to

mimic the effect or activity of a Wnt protein binding to a frizzled protein and/or LRP5 or LRP6. The ability of the Wnt surrogate molecules and other Wnt agonists disclosed herein to mimic the activity of Wnt can be confirmed by a number of assays. Wnt agonists typically initiate a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand. In particular, the Wnt agonists disclosed herein activate, enhance or increase the canonical Wnt/β-catenin signaling pathway. As used herein, the term "enhances" refers to a measurable increase in the level of Wnt/β-catenin signaling compared with the level in the absence of a Wnt agonist, e.g., a Wnt surrogate molecule disclosed herein. In particular embodiments, the increase in the level of Wnt/β-catenin signaling is at least 10%, at least 20%, at least 50%, at least two-fold, at least five-fold, at least 10-fold, at least 20-fold, at least 50-fold, or at least 100-fold as compared to the level of Wnt/β-catenin signaling in the absence of the Wnt agonist, e.g., in the same cell type. Methods of measuring Wnt/β-catenin signaling are known in the art and include those described herein.

In particular embodiments, Wnt surrogate molecules disclosed herein are bispecific, i.e., they specifically bind to two or more different epitopes, e.g., one or more Fzd receptor, and LRP5 and/or LRP6.

In particular embodiments, Wnt surrogate molecules disclosed herein are multivalent, e.g., they comprise two or more regions that each specifically bind to the same epitope, e.g., two or more regions that bind to an epitope within one or more Fzd receptor and/or two or more regions that bind to an epitope within LRP5 and/or LRP6. In particular embodiments, they comprise two or more regions that bind to an epitope within one or more Fzd receptor and two or more regions that bind to an epitope within LRP5 and/or LRP6. In certain embodiments, Wnt surrogate molecules comprise a ratio of the number of regions that bind one or more Fzd receptor to the number of regions that bind LRP5 and/or LRP6 of or about: 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 2:3, 2:5, 2:7, 7:2, 5:2, 3:2, 3:4, 3:5, 3:7, 3:8, 8:3, 7:3, 5:3, 4:3, 4:5, 4:7, 4:9, 9:4, 7:4, 5:4, 6:7, 7:6, 1:2,

1:3, 1:4, 1:5, or 1:6. In certain embodiments, Wnt surrogate molecules are bispecific and multivalent.

Wnt surrogate molecules disclosed herein may have any of a variety of different structural formats or configurations. Wnt surrogate molecules may comprise polypeptides and/or non-polypeptide binding moieties, e.g., small molecules. In particular embodiments, Wnt surrogate molecules comprise both a polypeptide region and a non-polypeptide binding moiety. In certain embodiments, Wnt surrogate molecules may comprise a single polypeptide, or they may comprise two or more, three or more, or four or more polypeptides. In certain embodiments, one or more polypeptides of a Wnt surrogate molecule are antibodies or antigen-binding fragments thereof. In certain embodiments, Wnt surrogates comprise two antibodies or antigen binding fragments thereof, one that binds one or more Fzd and one that binds LRP5 and/or LRP6. In certain embodiments, the Wnt surrogates comprises one, two, three, or four polypeptides, e.g., linked or bound to each other or fused to each other.

When the Wnt surrogate molecules comprise a single polypeptide, they may be a fusion protein comprising one or more Fzd binding domain and one or more LRP5/6 binding domain. The binding domains may be directly fused or they may be connected via a linker, e.g., a polypeptide linker, including but not limited to any of those disclosed herein.

When the Wnt surrogate molecules comprise two or more polypeptides, the polypeptides may be linked via covalent bonds, such as, e.g., disulfide bonds, and/or noncovalent interactions. For example, heavy chains of human immunoglobulin IgG interact at the level of their CH3 domains directly, whereas, at the level of their CH2 domains, they interact via the carbohydrate attached to the asparagine (Asn) N84.4 in the DE turn. In particular embodiments, the Wnt surrogate molecules comprise one or more regions derived from an antibody or antigen-binding fragment thereof, e.g., antibody heavy chains or antibody light chains or fragments thereof. In certain embodiments, a Wnt surrogate polypeptide comprises two antibody heavy chain regions (e.g., hinge regions) bound together via one or more disulfide

bond. In certain embodiments, a Wnt surrogate polypeptide comprises an antibody light chain region (e.g., a C_L region) and an antibody heavy chain region (e.g., a C_H1 region) bound together via one or more disulfide bond.

Wnt surrogate polypeptides may be engineered to facilitate binding between two polypeptides. For example, Knob-into-holes amino acid modifications may be introduced into two different polypeptides to facilitate their binding. Knobs-into-holes amino acid (AA) changes is a rational design strategy developed in antibody engineering, used for heterodimerization of the heavy chains, in the production of bispecific IgG antibodies. AA changes are engineered in order to create a knob on the CH3 of the heavy chains from a first antibody and a hole on the CH3 of the heavy chains of a second antibody. The knob may be represented by a tyrosine (Y) that belongs to the 'very large' IMGT volume class of AA, whereas the hole may be represented by a threonine (T) that belongs to the 'small' IMGT volume class. Other means of introducing modifications into polypeptides to facilitate their binding are known and available in the art. For example, specific amino acids may be introduced and used for cross-linking, such as Cysteine to form an intermolecular disulfide bond.

Wnt surrogate molecules may have a variety of different structural formats, including but not limited to those shown in FIG. 1.

In one embodiment, a Wnt surrogate molecule comprises an scFv or antigen-binding fragment thereof fused to a VHH or sdAb or antigen-binding fragment thereof. In certain embodiments, the scFv specifically binds one or more Fzd receptor, and the VHH or sdAb specifically binds LRP5 and/or LRP6. In certain embodiments, the scFv specifically binds LRP5 and/or LRP6, and the VHH or sdAb specifically binds one or more Fzd receptor. In particular embodiments, the scFv or antigen-binding fragment thereof is fused directly to the VHH or sdAb or antigen-binding fragment thereof, whereas in other embodiments, the two binding regions are fused via a linker moiety. In particular embodiments, the VHH or sdAb is fused to the N-terminus of the scFV, while in other embodiments, the VHH or sdAb is fused to the C-terminus

of the scFv. In particular embodiments, the scFv is described herein or comprises any of the CDR sets described herein. In particular embodiments, the VHH or sdAb is described herein or comprises any of the CDR sets disclosed herein.

In various embodiments, including but not limited to those depicted in FIG. 1A, a Wnt surrogate molecule comprises one or more Fab or antigen-binding fragment thereof and one or more VHH or sdAb or antigen-binding fragment thereof (or alternatively, one or more scFv or antigen-binding fragment thereof). In certain embodiments, the Fab specifically binds one or more Fzd receptor, and the VHH or sdAb (or scFv) specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds LRP5 and/or LRP6, and the VHH or sdAb (or scFv) specifically binds one or more Fzd receptor. In certain embodiments, the VHH or sdAb (or scFv) is fused to the N-terminus of the Fab, while in some embodiments, the VHH or sdAb (or scFv) is fused to the C-terminus of the Fab. In particular embodiments, the Fab is present in a full IgG format, and the VHH or sdAb (or scFv) is fused to the N-terminus and/or C-terminus of the IgG light chain. In particular embodiments, the Fab is present in a full IgG format, and the VHH or sdAb (or scFv) is fused to the N-terminus and/or C-terminus of the IgG heavy chain. In particular embodiments, two or more VHH or sdAb (or scFvs) are fused to the IgG at any combination of these locations.

Fabs may be converted into a full IgG format that includes both the Fab and Fc fragments, for example, using genetic engineering to generate a fusion polypeptide comprising the Fab fused to an Fc region, i.e., the Fab is present in a full IgG format. The Fc region for the full IgG format may be derived from any of a variety of different Fcs, including but not limited to, a wild-type or modified IgG1, IgG2, IgG3, IgG4 or other isotype, e.g., wild-type or modified human IgG1, human IgG2, human IgG3, human IgG4, human IgG4Pro (comprising a mutation in core hinge region that prevents the formation of IgG4 half molecules), human IgA, human IgE, human IgM, or the modified IgG1 referred to as IgG1 LALAPG. The $L_{235}A$, $P_{329}G$ (LALA-PG)

variant has been shown to eliminate complement binding and fixation as well as Fc-γ dependent antibody-dependent cell-mediated cytotoxity (ADCC) in both murine IgG2a and human IgG1. These LALA-PG substitutions allow a more accurate translation of results generated with an "effectorless" antibody framework scaffold between mice and primates. In particular embodiments of any of the IgG disclosed herein, the IgG comprises one or more of the following amino acid substitutions: N297G, N297A, N297E, L234A, L235A, or P236G.

Non-limiting examples of bivalent and bispecific Wnt surrogate molecules that are bivalent towards both the one or more Fzd receptor and the LRP5 and/or LRP6 are provided as the top four structures depicted in FIG. 1A, where the VHH or sdAb or scFv is depicted in white, and the Fab or IgG is depicted in black. As shown, the VHH or sdAb (or scFvs) may be fused to the N-termini of both light chains, to the N-termini of both heavy chains, to the C-termini of both light chains, or to the C-termini of both heavy chains. It is further contemplated, e.g., that VHH or sdAb (or scFvs) could be fused to both the N-termini and C-termini of the heavy and/or light chains, to the N-termini of the light chains and the heavy chains, to the C-termini of the heavy and light chains, to the N-termini of the heavy chains and C-termini of the light chains, or to the C-termini of the heavy chains and the N-termini of the light chains. In other related embodiments, two or more VHH or sdAb (or scFvs) may be fused together, optionally via a linker moiety, and fused to the Fab or IgG at one or more of these locations. In a related embodiment, the Wnt surrogate molecule has a Hetero-IgG format, whereas the Fab is present as a half antibody, and one or more VHH or sdAb (or scFv) is fused to one or more of the N-terminus of the Fc, the N-terminus of the Fab, the C-terminus of the Fc, or the C-terminus of the Fab. A bispecific but monovalent to each receptor version of this format is depicted at the bottom of FIG. 1A. In certain embodiments, the Fab or antigen-binding fragment (or IgG) thereof is fused directly to the VHH or sdAb (or scFv) or antigen-binding fragment thereof, whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments,

the Fab is described herein or comprises any of the CDR sets described herein. In particular embodiments, the VHH or sdAb or scFv is described herein or comprises any of the CDR sets disclosed herein.

In various embodiments, including but not limited to those depicted in FIG. 1B, a Wnt surrogate molecule comprises one or more Fab or antigen-binding fragment thereof that binds one or more Fzd receptor and one or more Fab or antigen-binding fragment thereof that binds LRP5 and/or LRP6. In certain embodiments, it comprises two Fab or antigen-binding fragments thereof that bind one or more Fzd receptor and/or two Fab or antigen-binding fragments thereof that bind LRP5 and/or LRP6. In particular embodiments, one or more of the Fab is present in a full IgG format, and in certain embodiments, both Fab are present in a full IgG format. In certain embodiments, the Fab in full IgG format specifically binds one or more Fzd receptor, and the other Fab specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds one or more Fzd receptor, and the Fab in full IgG format specifically binds LRP5 and/or LRP6. In certain embodiments, the Fab specifically binds LRP5 and/or LRP6, and the Fab in full IgG format specifically binds one or more Fzd receptor. In certain embodiments, the Fab is fused to the N-terminus of the IgG, e.g., to the heavy chain or light chain N-terminus, optionally via a linker. In certain embodiments, the Fab is fused to the N-terminus of the heavy chain of the IgG and not fused to the light chain. In particular embodiments, the two heavy chains can be fused together directly or via a linker. An example of such a bispecific and bivalent with respect to both receptors is shown at the top of FIG. 1B. In other related embodiments, two or more VHH or sdAb may be fused together, optionally via a linker moiety, and fused to the Fab or IgG at one or more of these locations. In a related embodiment, the Wnt surrogate molecule has a Hetero-IgG format, whereas one of the Fab is present as a half antibody, and the other Fab is fused to one or more of the N-terminus of the Fc, the N-terminus of the Fab, or the C-terminus of the Fc. A bispecific but monovalent to each receptor version of this format is depicted at the bottom of FIG. 1B. In certain embodiments, the Fab or

antigen-binding fragment thereof is fused directly to the other Fab or IgG or antigen-binding fragment thereof, whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the one or both of the two Fabs are described herein or comprise any of the CDR sets described herein.

In certain embodiments, Wnt surrogate molecules have a format as described in PCT Application Publication No. WO2017/136820, e.g., a Fabs-in-tandem IgG (FIT-IG) format. Shiyong Gong, Fang Ren, Danqing Wu, Xuan Wu & Chengbin Wu (2017). FIT-IG also include the formats disclosed in "Fabs-in-tandem immunoglobulin is a novel and versatile bispecific design for engaging multiple therapeutic targets" mAbs, 9:7, 1118-1128, DOI: 10.1080/19420862.2017.1345401. In certain embodiments, FIT-IGs combine the functions of two antibodies into one molecule by re-arranging the DNA sequences of two parental monoclonal antibodies into two or three constructs and co-expressing them in mammalian cells. Examples of FIT-IG formats and constructs are provided in FIGS. 1A and 1B and FIGS. 2A and 2B of PCT Application Publication No. WO2017/136820. In certain embodiments, FIT-IGs require no Fc mutation; no scFv elements; and no linker or peptide connector. The Fab-domains in each arm work "in tandem" forming a tetravalent bi-specific antibody with four active and independent antigen binding sites that retain the biological function of their parental antibodies In particular embodiments, Wnt surrogates comprises a Fab and an IgG. In certain embodiments, the Fab binder LC is fused to the HC of the IgG, e.g., by a linker of various length in between. In various embodiment, the Fab binder HC can be fused or unfused to the LC of the IgG. A variation of this format has been called Fabs-in-tandem IgG (or FIT-Ig).

In particular embodiments, Wnt surrogate molecules comprise two or more VHH or sdAb (or scFvs), including at least one that binds one or more Fzd receptor and at least one that binds LRP5 and/or LRP6. In certain embodiments, one of the binding regions is a VHH or sdAb and the other is an scFv. Wnt memetic molecules comprising two or more VHH or sdAb (or scFvs)

may be formatted in a variety of configurations, including but not limited to those depicted in FIG. 1C. In certain bispecific, bivalent formats, two or more VHH or sdAb (or scFvs) are fused in tandem or fused to two different ends of an Fc, optionally via one or more linkers. Where linkers are present, the linker and its length may be the same or different between the VHH or sdAb (or scFv) and the other VHH or sdAb (or scFv), or between the VHH or sdAb and Fc. For example, in certain embodiments, the VHH or sdAb is fused to the N-terminus and/or C-terminus of the IgG heavy chain. In particular embodiments, two or more VHH or sdAb are fused to the IgG at any combination of these locations. Non-limiting examples of bivalent and bispecific Wnt surrogate molecules of this format are depicted as the top seven structures depicted in FIG. 1C, where the first VHH or sdAb is depicted in white, the Fc or IgG is depicted in black, and the second VHH or sdAb is depicted as light gray. In various embodiments, both VHH or sdAb may be fused to the N-termini of the Fc, to the C-termini of the Fc, or one or more VHH or sdAb may be fused to either or both of an N-terminus or C-terminus of the Fc. In a related embodiment, the Wnt surrogate molecule has a Hetero-IgG format, whereas one VHH or sdAb is present as a half antibody, and the other is fused to the N-terminus of the Fc or the C-terminus of the Fc. A bispecific but monovalent to each receptor version of this format is depicted at the bottom of FIG. 1C. In certain embodiments, the VHH or sdAb is fused directly to the other VHH or sdAb whereas in other embodiments, the binding regions are fused via a linker moiety. In particular embodiments, the VHH or sdAb are described herein or comprises any of the CDR sets described herein. In various embodiments, any of these formats may comprise one or more scFvs in place of one or more VHH or sdAb.

In certain embodiments, a Wnt surrogate molecule is formatted as a diabody. As shown in Fig. 1D, the binders against Fzd and LRP can also be linked together in a diabody (or DART) configuration. The diabody can also be in a single chain configuration. If the diabody is fused to an Fc, this will create a bivalent bispecific format. Without fusion to Fc, this would be a monovalent bispecific format. In certain embodiments, a diabody is a noncovalent dimer

scFv fragment that consists of the heavy-chain variable (VH) and light-chain variable (VL) regions connected by a small peptide linker. Another form of diabody is a single-chain (Fv)2 in which two scFv fragments are covalently linked to each other.

As discussed, Wnt surrogate molecules, in various embodiments, comprise one or more antibodies or antigen-binding fragments thereof disclosed herein. Thus, in particular embodiments, a Wnt surrogate comprises two polypeptides, wherein each polypeptide comprises an Nab or scFv that binds LRP5/6 and an Nab or scFv that binds one or more Wnts, optionally wherein one of the binding domains is an scFv and the other is an Nab. In certain embodiments, a Wnt surrogate comprises three polypeptides, wherein the first polypeptide comprises an antibody heavy chain and the second polypeptide comprises an antibody light chain, wherein the antibody heavy chain and light chain bind LRP5/6 or one or more Fzds, and wherein the third polypeptide comprises a VHH or sdAb fused to a heavy chain Fc region, wherein the VHH or sdAb binds to either LRP5/6 or one or more Fzds. In other embodiments, Wnt polypeptides comprise four polypeptides, including two heavy chain polypeptides and two light chain polypeptides, wherein the two heavy chains and two light chains bind LRP5/6 or one or more Fzds, and further comprise one or more Nab or scFv fused to one or more of the heavy chains and/or light chains, wherein the Nab or scFv binds to LRP5/6 or one or more Fzds. In another illustrative embodiment, a Wnt surrogate comprises at least four polypeptides, including two heavy chain polypeptides and two light chain polypeptides that bind either LRP5/6 or one or more Fzds, wherein the Wnt surrogate further comprises a Fab that binds either LRP5/6 or one or more Fzds. For example, the Fab may comprise two polypeptides, each fused to one of the two heavy chain polypeptides, and two polypeptides, each fused to one of the two light chain polypeptides, or it may comprise two polypeptides each fused to one of the two heavy chain polypeptides and two additional polypeptides, each bound to one of the two polypeptides fused to the heavy

chain polypeptides, thus making a second Fab. Other configurations may be used to produce the Wnt surrogates disclosed herein.

In particular embodiments, a Wnt surrogate molecule comprises a Fzd binding region, e.g., an anti-Fzd antibody, or antigen-binding fragment thereof, fused or bound to a polypeptide that specifically binds to one or more Fzd receptor. In particular embodiments, the polypeptide that specifically binds to one or more Fzd receptor is an antibody or antigen-binding fragment thereof. If certain embodiments, it is an antibody or antigen-binding fragment thereof disclosed herein or in the U.S. provisional patent application no. 62/607,877, titled, "Anti-Frizzled antibodies and Methods of Use," Attorney docket number SRZN-004/00US, filed on December 19, 2017, which is incorporated herein by reference in its entirety. In particular embodiments, the Fzd binding domain comprises the three heavy chain CDRs and/or the three light chain CDRs disclosed for any of the illustrative antibodies or fragments thereof that bind to one or more Fzd receptor provided in Table 1A. In particular embodiments, the Fzd binding domain comprises the three heavy chain CDRs and/or the three light chain CDRs disclosed for any of the illustrative antibodies or fragments thereof that bind to one or more Fzd receptor provided in Table 1A, wherein the CDRs collectively comprise one, two, three, four, five, six, seven, or eight amino acid modifications, e.g., substitutions, deletions, or additions. In certain embodiments, the Fzd binding domain is a VHH or sdAb or was derived from a VHH or sdAb, so Table 1A only includes the three heavy chain CDRs. In particular embodiments, the Fzd binding domain comprises the three CDR HC sequences provided in Table 1A or variants wherein the CDRs collectively comprise one, two, three, four, five, six, seven or eight amino acid modifications. In particular embodiments, the Fzd binding domain comprises the heavy chain fragment and/or light chain fragment of any of the illustrative antibodies or fragments thereof that bind to one or more Fzd receptor provided in Table 1B or SEQ ID NOs:1-65 or 129-132 (or an antigen-binding fragment or variant of either). In certain embodiments, the Fzd binding domain is an Fab or was derived from an Fab, so the heavy chain of Table 1B includes VH and CH1

sequence, but not CH2 or CH3 sequences. In certain embodiments, the Fzd binding domain is a VHH or sdAb or was derived from a VHH or sdAb, so Table 1B includes the VHH domain. In certain embodiments, the Fzd binding region is a polypeptide, e.g., an antibody or antigen-binding fragment thereof, that competes with any of these antibodies for binding to one or more Fzd receptor.

Table 1A: Anti-Fzd Antibody Clone IDs and CDR sequences

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
001S-A01	Fzd1	YTFTSYGIS	391	GWISAYNG NTNYA	570	CARASAWT PYGAFDIW	752	SGSSSNIGS HTVS	1156	SNYQRPS	1256	CAAWDGS LFGHWVF	1265
001S-B01	Fzd1	GSISSGGYS WS	283	GSIYHSGST YYN	547	CARFYYDILT GYSYFDYW	818	RSSRSLLDT DDGNTYLD	1142	TLSHRAS	1259	CMQSIQLP WTF	1295
001S-E01	Fzd1	GSISNYYWS	282	GEIDRSGD TNYN	488	CARVRARRF LVSDRSAFD IW	945	SGNTLGSHY VS	1155	QDSKRPS	1246	CQVWDSS TVVF	1431
001S-F01	Fzd1	GSISGNNYY ZG	281	GSIYFTGGT YYN	546	CARVMLITD AFDIW	942	RSSQSLLHS NGYNYLD	1138	LGSNRAS	1237	CMQGTHW PYTF	1289
001S-G01	Fzd1	GSISSSSYY WG	285	GYIYYSGST YYN	589	CARATYGG DAFDIW	760	TRSSSNIGA GYDVH	1161	GNSIRPS	1220	CGTWDSS LSAWVF	1267
001S-H01	Fzd1	GSISSGGYY WS	284	GYIYYSGST YYN	589	CARHAGFY GLADYFDY W	875	RSSQSLLHS NGYNYLD	1138	LGSKRAS	1236	CMQALQIP PTF	1280
001S-A02	Fzd1	GSISSGGYY WS	284	GYIYYSGST YYN	589	CARGKGYS YGYGKDWF DPW	845	QASQDIGKY LN	1041	DASNLET	1185	CQQNDYLP LTF	1332
001S-E02	Fzd1	GSISGNNYY WG	280	GSIYFTGGT YYN	546	CARVMLITD AFDIW	942	RSSQSLLHS NGYNYLD	1138	LGSNRAS	1237	CMQGTHW PYTF	1289
001S-G02	Fzd1	GAISGTSYF WG	266	GSIYYTGNT YYN	548	CARIGIAVAA PVDHW	882	RASQSVGTY LT	1110	DASNRAT	1188	CMQATQFP LTF	1284
001S-H02	Fzd1	GSISSSSYY WG	285	GYIYYSGST YYN	589	CARATYGG DAFDIW	760	TRSSSNIGA GYDVH	1161	GNSIRPS	1220	CGTWDSS LSAWVF	1267
001S-A03	Fzd1	GSISSGGYY WS	284	GYIYYSGST YYN	589	CARVRDYY DSSGYYYDY FDYW	946	RASRSISSYF N	1128	AASSLQS	1175	CQQADTFP PTF	1314
001S-B03	Fzd1	ASFSGHYW T	158	GEIDHTGST NYE	487	CARGGQGG YDWGHYHG LDVW	835	SGDKVGHKY AS	1154	EDSQRPS	1199	CQAWDSS TDVVF	1301
001S-H08	Fzd5	RAFTDNVM A	329	ATISGGGG STFDD	466	CAAASSLTS TPYDLW	678						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
001S-A09	Fzd5	RSFRTNALG	333	AAISWTGG STYYA	422	CNTVTYTGG SYKNYW	1005						
001S-B09	Fzd5	SIDSINAMA	356	AALTSGGIT YHA	428	CNVITIVRG MGPRAYW	1006						
001S-C09	Fzd5	SIFSINAMG	357	ATIQSGGRT NYA	465	CNVITIVRG MGPRAYW	1006						
001S-C07	Fzd8	YTFTSYGIS	391	GWISAYNG NTNYA	570	CARDGTPFY SGSYYGSW	772	QGDSLRTYY AS	1052	GKNNRPS	1219	CNSRDNS GKHVF	1300
001S-D07	Fzd8	GTFSSYAIS	295	GRIIPILGIA NYA	529	CARVPTSPY DILTGPFDY W	944	RSSQSLLHS NGFNYVD	1136	FGSYRAS	1206	CMQNLQT PWTF	1291
001S-E07	Fzd8	ASVSSNSAA WN	159	GRTYYRSK WYNDYA	542	CARWKNYF DPW	953	RASQGIRSD LA	1070	AASTLES	1177	CLQDYSYP RTF	1273
001S-H07	Fzd8	FTFSSYAMS	228	STISGGGG STYYA	646	CAKDLVPW GSSAFNIW	704	RASQSVSSY LA	1121	GASSRAT	1213	CQQYGSS PPTF	1410
004S-E05	Fzd5	FTFSTYEMN	243	SGVSWNGS RTHYV	618	CARGQSEK WWSGLYGM DVW	856	RASQGISSA LA	1076	AASALQS	1165	CQQTYSTP RTF	1394
004S-E03	Fzd5	GTFSTYAIS	298	GWINSGNG NTKYS	565	CWTGLLWF GESTDAFDI W	1031	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-G06	Fzd5	GTFTYRYLH	307	GGIIPIFGTG NYA	501	CASSMVRV PYYYGMDV W	964	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
001S-D09	Fzd8	GPFNLFAM G	272	AGISRTGG NTGYA	445	CASKTTINS GWSREYHY W	958						
001S-E09	Fzd8	GPFNLFAM G	272	AGISRTGG NTGYA	445	CASKTTINS GWSREYHY W	958						
001S-F09	Fzd8	GFFSSFTMG	268	AAISRNGVY TRFA	409	CNALAPGVR GSW	987						
001S-G09	Fzd8	SLFRLNGM G	360	ATISTRGTT HYA	467	CTDEESW	1011						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
001S-H09	Fzd8	GPFNLLAMG	273	AGISRTGGNTGYA	445	CASKTTINSGWSREYHYW	958						
001S-A10	Fzd8	SVVNFVVMG	364	AAITSGGSTNYA	425	CNRVGSREYSYW	1001						
001S-B10	Fzd8	RTSDLYTMG	352	AAIGYKVKWNGERTYYL	404	CNAVTYNGYTIW	994						
001S-G12	Fzd1	SIFSSNTIY	359	ALITTSGNTNYA	455	CNAGAPAWTYRMGTYYPQFGSW	986						
002S-A01	Fzd1	STFSTYAMG	362	AAISGSGENTYYA	408	CVKFGMNLGYSGYDYW	1028						
002S-B01	Fzd1	STFSNYAMG	361	AAISWGGGSTFYS	411	CAAGPIARWYRGDMDYW	681						
002S-C01	Fzd1	RMFSNYAMG	331	AAISSGGSGTYYS	410	CAAGPIARWYRGDMDYW	681						
002S-D01	Fzd1	RTDGGYVMG	337	ATVTWRTGTTYYA	469	CAAGPIARWYRGDMDYW	681						
002S-E01	Fzd1	RTFSSAAMG	345	AAISWSGSTAYYA	421	CATLTPYGTVASY	974						
002S-F01	Fzd1	RTFSSYAMG	347	AAVNWSGGSTYYA	430	CAAVFLSRNYEIQEYYRYQ	689						
002S-G01	Fzd1	RTFSSYAMG	347	AAISWSGGSTYYA	418	CAAGPIARWYRGDMDYW	681						
002S-H01	Fzd1	RSFSTYPMG	336	TVISGSGGSTYYS	676	CAAGPIARWYRGDMDYW	681						
002S-A02	Fzd1	RRFTTYGMG	332	AAVTWRSGSTYYA	436	CYLEGPLDVYW	1032						
002S-B02	Fzd1	RTFNRHVMG	341	AAISWSGDSTYYA	415	CAKLGGSSWLREYDYW	724						
002S-C02	Fzd1	RTFRAYAMG	342	SAISWSGGSTYYA	603	CAAGPIARWYRGDMDYW	681						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
002S-D02	Fzd1	RTFSEYAM G	343	AAISWSGG STHYA	417	CNADSLRGI DYW	984						
002S-E02	Fzd1	FTFREYAMT	199	SGISRDGG RTSYS	613	CAPRVLVTA PSGGMDYW	734						
002S-F02	Fzd1	GDFTNYAM A	267	AAVNWRGD GTYYS	429	CAAVFLSRN YEIQEYYRY Q	689						
002S-G02	Fzd1	RTFGTWAM G	340	AAISYNGFS TYYS	424	CAAGPIARW YRGDMDYW	681						
002S-H02	Fzd1	RTFSSYAM G	347	AAISWSGG STYYA	418	CAAGPIARW YRGDMDYW	681						
002S-D03	Fzd1	RTFGSYAM G	339	AAISWSGG STYYA	418	CAAGPIARW YRGDMDYW	681						
002S-E03	Fzd1	SIFSIYAMG	358	AVVATGGA TNYA	481	CNMRGNWY REGRPAEFL SW	1000						
002S-F03	Fzd1	RTSSSYAM G	353	AAISWSGG STYYA	418	CAAGPIARW YRGDMDYW	681						
002S-G03	Fzd1	RTFGSYAM G	339	AAISWSGG STYYA	418	CAAGPIARW YRGDMDYW	681						
002S-H03	Fzd1	QTFTAYAM G	327	AAISWSGS ATHYA	420	CNAWWLVA GSRGTSAD YW	996						
002S-A04	Fzd1	RTFSSYAM G	347	AAISWSGR STYYA	419	CAAGPIARW YRGDMDYW	681						
002S-B04	Fzd1	RTFSSYAM G	347	AAISWSGG STYYA	418	CAAGPNYS WFMPSSSR LIW	662						
002S-C04	Fzd1	RRFTTYGM G	332	AAVTWRAG STYYA	435	CSADKLDYL DDQPFKTW DYW	1010						
002S-D04	Fzd1	GTSSTYAM G	309	AAINRSGG STYYA	405	CAAVFLSRN YEIQEYYRY Q	669						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
002S-E04	Fzd1	GTFSTYAM G	300	AAISWSGD STYYL	416	CAAGPIARW YRGDMDYW	681						
004S-H04	Fzd5	GTFSSYAIS	295	GWISTYNG ATNYA	577	CARGGAGR FGEGMDVW	826	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
001S-A04	Fzd5	YTFTSYGIS	391	GWISAYNG NTNYA	570	CASSKEKAT YYYGMDVW	963	GLSSGSVST NYYPS	1035	YTNTRSS	1263	CLLYLGRGI WVF	1271
001S-D03	Fzd5	GTFSSYAIS	295	GRIIPILGIA NYA	529	CARLDPGYY YGMDVW	866	TGTSSDVGG YNSVS	1159	DVTKRPS	1196	CFSYAGSR F	1266
001S-F03	Fzd5	GTFSSYAIS	295	GGIIPIFGTA NYA	499	CARVIFSTVT TTNDIW	939	TRSSGSIAS NYVQ	1160	ENDKRPS	1202	CQSYDYD HRWVF	1430
004S-E04	Fzd5	YTFSGYYLH	374	GTVTPILGT ANYA	549	CARVDGSG YYGIDYW	933	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-A06	Fzd5	GSFSNYAIS	278	GRIIPILGSA NYA	530	CARTYLKAF DIW	930	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F04	Fzd5	YTFTNNFMH	383	GRINPNSG GTNYA	537	CARDRFDN WFDPW	786	RASQGISSA LA	1076	AASTLQS	1179	CQQSYNTP WTF	1351
001S-C03	Fzd5	GTFSSYAIS	295	GRIIPILGIA NYA	529	CAREGRSR VYGGNSFD YW	806	RSSQSLLRR NGHNYVD	1139	MGSNRAP	1238	CMHGLHP PFTF	1279
003S-A01	Fzd1	YIFTDYYMH	368	GGIIPIFGTA NYA	499	CARMSSDY YDSSGYYR RGMDVW	895	RASQGISNN LN	1072	GASTLQS	1215	CQQADSFP PTF	1312
003S-E01	Fzd1	YIFTDYYMH	368	GGIIPIFGTA NYA	499	CARAWKGL WFGEGTFD YW	761	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-F01	Fzd1	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARLAFDIW	865	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-A02	Fzd1	YTFTGYYMH	379	GWINAGNG NTTYA	558	CAKDRGNY GDYLDYW	707	RASQGISNY LA	1074	EVSSVQG	1204	CQQSYSTP LAF	1370
003S-C02	Fzd1	FTFSNSDMN	214	ALISYDGSH TYYA	454	CTRGSRIGW FDPW	1015	RASQSIGRW LA	1084	AASRLQS	1171	CQQGFNFP LTF	1325
003S-E02	Fzd1	GTFSSYTIS	296	GGIIPISGKT DYA	505	CARARGGD SPLSL	749	RASQGISNN LN	1072	TASSLQS	1258	CLQDYSYP YTF	1274

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
003S-F02	Fzd1	GTFRSYAIN	292	GGIIPIFGTA NYA	499	CARGGWRP DYYGSGSY YSFDYW	840	RASQSVSSD LA	1115	GASTRAT	1217	CQQYETW PVLTF	1405
003S-G02	Fzd1	FTFGTYWVT	196	SGITGSGG RTFYA	616	CARMKDWF GAFDIW	894	RASESVSSS SFA	1056	GASTRAT	1217	CQQYNNW PPNYTF	1420
003S-C03	Fzd1	FTFSRYAMS	220	SYISGDSGY TNYA	658	CARGLVIAT NWFDPW	849	QANQDISNY LN	1038	AASSLQS	1175	CQQTYNPP RTF	1389
003S-D03	Fzd1	YTFTSYYMH	392	GWINTYNG NTNYP	567	CAESLTSTA DW	691	RASQGISNN LN	1072	AASSLQR	1174	CQQSYSTP FTF	1366
003S-E03	Fzd1	YIFTDYYMH	368	GWWNPTTG NTGYA	586	CARNVEGAT SFPEFDYW	896	RASQGISNN LN	1072	SASNLQS	1252	CQQSYSPP PYTF	1364
003S-H03	Fzd1	GTFSSYAIS	295	GGIIPIFGTA NYA	499	CAKDIGSSW YYYMDVW	701	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-A04	Fzd1	FTFGTYWVT	196	SGITGSGG RTFYA	616	CARMKDWF GAFDIW	894	RTSERSSISS FA	1148	GASTRAT	1217	CQQYNNW PRNYTF	1420
003S-C04	Fzd1	FAVSSSYMS	168	ASIWFDGS NQDYA	463	CAPNESGN VDYW	733	RASQGISNN LN	1072	KASSLEN	1225	CQQSYSTP HTF	1369
003S-D04	Fzd1	FTFSSYAMH	227	SAISGSGG STYYA	600	CARDHGSS WYQNTDAF DIW	774	QASQDIGNY LN	1042	DVSNLER	1195	CQHLNSYP PGDTF	1304
003S-G04	Fzd1	FRFISHPIH	177	GRVIPILGV TNYA	545	CASSSDYG DYLKEPNYG MDVW	966	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-D05	Fzd2	FTFSNYAMT	216	SAIGTGGG TYYA	595	CATAYRRPG GLDVW	969	RSSQSLLHS DGKTYLY	1134	LGSNRAS	1237	CMQNTHW PLTR	1293
003S-E05	Fzd2	FTFSSYTMS	236	GRIKSKAN GGTTDYA	535	CARGSSSW YDW	863	KSSQSLLHS DGKTYLY	1036	LGSNRAS	1237	CMQNTHW PLTR	1293
003S-A06	Fzd2	FTFADYGM H	188	SYISSGSYT IYYS	659	CARGTFDW LLSPSYDYW	865	RASQGISNN LN	1072	AASRLES	1170	CQQSYSTP LTF	1372
003S-C06	Fzd2	FTFSNYGM H	217	SAISNSGGS TYYA	601	CTSSFLTGS QPSGYW	1018	RASQDISSY LA	1065	AASSLQS	1175	CQQSYRTP LTF	1353
003S-G06	Fzd2	FTFSDYGM H	207	SSTSGSGG NSKYS	642	CARHNPGY MGYYYGMD VW	877	RASQSVSSN LA	1116	DASNRAT	1188	CQHRTSW PLTF	1307

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
003S-H06	Fzd2	GTFSSYTIS	296	GLVDPEDG ETIYA	520	CTILPAAAA GTYYYYGM DVW	1012	RASQRVGN NLA	1083	DASIRAT	1184	CQQYKDW PTF	1415
003S-B07	Fzd2	FTFSDHYMS	205	SSITRTPSG GTTEYA	639	CARDGGYW	768	RASQSVGSY LA	1109	GSSNRAA	1221	CQQYGTSL LTF	1414
003S-D07	Fzd2	YTFTNNFMH	383	GIINPSGGS TSYA	513	CARATSLGR RYCSSTSCY PRDAFDIW	759	QASQGISNN LN	1049	LGSDRAS	1233	CQQSYSTP FTF	1368
003S-E07	Fzd2	YTFTNNFMH	383	GWINPNSG GTKYA	563	CARSVGEV GATMLGIGV WYWFDPW	926	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP FTF	1368
003S-A08	Fzd2	FTFSNYAMT	216	SAIGTGGG TYYA	595	CATAYRRPG GLDVW	969	RSSQSLLHS DGKTYLY	1134	LGFNRAS	1232	CMQNTHW PLTR	1293
003S-C08	Fzd2	LTVSTNFMS	324	AGIGWDST NIGYA	440	CARDLVAAR PSNWDYW	782	RASQGIRND LG	1069	GASTLQR	1214	CQQSYSTP RVTF	1374
003S-E08	Fzd2	FTFRNSAMH	201	STISGSGGS TYYS	647	CARGGGYS SSW	829	RSSRSLLHS DGKTYLY	1143	LGSNRAS	1237	CMQSSHW PKTF	1298
003S-G09	Fzd4	FTFDHNPM N	194	SAIGAGGG TYYA	593	CASPTVTRR	960	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-C10	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-D10	Fzd4	FNFGIYSMT	172	SYISGDSGY TNYA	658	CARVGPGG WFDPW	936	RASQGISSY LA	1076	AASNLLG	1167	CQQTYSTP WTF	1396
003S-E10	Fzd4	FTFSSYAMH	227	AGISASGG STYYA	442	CARPSTTGT KAFDIW	901	RASQSIGSN LD	1085	AASTLET	1178	CQQSYSVP DTF	1380
003S-A11	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	RASQSISZYZ N	1103	ZASSLQS	1264	CQQSYSTP LTF	1372
003S-G11	Fzd4	GTFSSYAIS	295	GRIIPIFGTV NYA	528	CARGARLDY W	820	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-H11	Fzd4	YTFTGYYMH	379	GGIIPIFGTP HYA	502	CASTDPSSG LDYW	967	RASQSIGSN LD	1085	DASSLES	1189	CQQSFIMP LTF	1341
003S-C12	Fzd4	GTFSSYAIS	295	GWINPNSG GTNYA	564	CARGGSSD VR	836	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
003S-F12	Fzd4	FTFSSYAMH	227	SVISTSGDT VLYT	652	CARGGSSD VR	838	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-B01	Fzd4	GTFSSYAIS	295	GIINPSGGS TSYA	513	CAKDGVVR	698	RAIQSISSYL N	1054	AASSLQS	1175	CQQSYSTP LTF	1372
004S-C01	Fzd4	FTFSNHYTS	213	STISSSGGR TFYA	650	CARASRIDG GWFIIDHL	754	RASQDIRDE LA	1062	AASTLQS	1179	CQQADSFP LTF	1311
004S-D01	Fzd4	FTFTNYAMS	248	SAISGSGG STYYA	600	CARATGFGT VVFDYW	757	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-E01	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	ZAQLRIISYL N	1163	FASSLQS	1205	CQQSYSTP LTF	1372
004S-F01	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARDGVE	773	RASQGISNW LA	1073	DASSLQS	1190	CQQSHITP YTF	1344
004S-H01	Fzd4	FTFSNYAMH	215	ALMSPDGTI IYYA	456	CAKGIVGDY GAFDIW	717	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-B02	Fzd4	FTFSSYGMH	230	SSINNSSRT VFYA	630	CAKDHLAVA DAHGR	700	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-E02	Fzd4	FTFSSYAMH	227	AVISYDGSN EYYA	474	CAGGEVYEL	692	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F02	Fzd4	FTFSTYAMH	242	AVISSDGNN KYYT	473	CAAPDVVVT ADGYYW	685	RASQGISSA LA	1076	AASTLQS	1179	CQQANTVP FTF	1322
004S-G02	Fzd4	FTFANYAMN	190	ALISYDGGT KYYA	453	CAKTLVTSH ALHIW	728	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-H02	Fzd4	FTFANYAMH	189	ALISYDGGN KYYA	452	CAKTLVTSH ALHIW	728	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
001S-E03	Fzd5	GSFSGYYW H	276	GEINHSGST NYN	469	CARGRRLV RFTVTSAFDI W	858	TGSSSNIGA GFGVH	1158	SDRNRPS	1255	CQSYDSSL RASVF	1429
001S-B05	Fzd5	GTFSSYAIS	295	GGIIPILGIA NYA	504	CARIPKPRG YSYGDNGS W	883	RSSQSLLHS NGNTYLD	1137	LGSDRTS	1234	CMQSLQTP YTF	1297
004S-A07	Fzd6	GNFKNYGIT	271	GRIIPALGT ANYA	525	CARQYCSG GSCYPDAFD IR	908	RASQDIRSA LA	1063	QASSLIS	1245	CQQSYSM PQTF	1361

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
004S-B07	Fzd6	FTFSSYSMN	233	GVISKDGD NKYYA	553	CASSRDGY NRLAFDIW	965	QASQDIRNY LN	1043	AASSLQS	1175	CQQSSRF WTF	1347
004S-A08	Fzd6	GTFSSYAIS	295	GRIIPILGIA NYA	529	CARDGGDY GMDVW	767	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-B08	Fzd6	YTFTNNFMH	383	GRINPNSG GTNYA	537	CASQNYYG SGSYPGFDY W	961	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-D08	Fzd6	YTFTYRYLH	394	GGIIPIFGTA NYA	499	CATHDSSGY YSFDYW	973	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-E08	Fzd6	FSVSSNYM N	187	SAIGTGGG TYYA	595	CTTRTYDSS GYYETQNYY MDVW	1024	RSSRSLLHS NGNTYLQ	1144	LGSNRAS	1237	CVQTTQSP LTF	1434
004S-G08	Fzd6	FTFSDYYMS	208	AAISYDESN KFYA	423	CARSAVAGA FDIW	916	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-A09	Fzd6	FTFRDYAMN	198	SGISWNSG SIGYA	615	CARRSGYS GSVYYYYG MDVW	913	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-B09	Fzd6	FTFSSFGMH	221	AGINWNGG SVVYA	441	CARGPSHQ HTFDIW	854	RASQGISSA LA	1076	AASSLQS	1175	CQQSYSHT AFTF	1357
004S-C09	Fzd6	YTFTNNFMH	383	GGFDPEDG ETIYA	492	CARVGRGY SFDYW	937	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-E09	Fzd6	DTFSNYVIS	163	GRISAYNG YKSYA	538	CARSSGYV GWFDPW	924	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F09	Fzd6	FTFSNYYTS	218	SYISGAGG STEYA	657	CARLPRRSG KGSAFDIW	888	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-H09	Fzd6	GTFSSYTIS	296	GWMNPNS GNTGYA	563	CARVGATSA GGMDVW	935	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-C10	Fzd6	YIFTDYYMH	368	GLVDPEDG ETIYA	520	CAHSDFFSG LSFGDW	693	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-D10	Fzd6	FTFSNSDMN	214	SSISTSGGS TYYA	637	CARGSYW	864	RASQNINNY LA	1081	RASTLQS	1249	CQQYSSYP YTI	1425
004S-E10	Fzd6	TTLNKYAIS	365	GRITPVVGV TNYA	539	CALSSSWY GGFDYW	731	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
004S-F10	Fzd6	GFTFSDHY	269	ALVGYDGS QQFYG	458	CNTGIPMLY W	1003	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-G10	Fzd6	FTFSDYYMS	208	SAISGSGFT YYA	599	CARVSRGFA FDYW	948	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-A11	Fzd6	GTFSSYAIS	295	GRIIPILGIA NYA	529	CARESVNNY YYMDVW	813	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-C11	Fzd6	FTFSSYAMH	227	ALTSYDGS KKFYA	457	CAKTGRGY AFDIW	726	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-E11	Fzd6	FTFSSYNMN	232	GRIKSKAN GGTTDYA	535	CAKAGQQL DW	696	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-H11	Fzd6	FTFTSSAMQ	249	GGIIPIFGTA NYA	499	CATVQTNYY DSSGRFSYR AHYFDYW	977	RASQSIRW LA	1094	AASSLQS	1175	CQQYVSYP LTF	1426
004S-A12	Fzd6	YTFTNNFMH	383	GRINPNSG GTNYA	537	CARGQGYS SGWYRGDA FDIW	855	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-D12	Fzd6	FAFDDYAM H	165	GFIRSKAYG GTTEYA	490	CAKDRGYS SGWYLDYW	708	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-H01	Fzd7	FNFSSYTMR	173	SVIYGGGN TNYA	653	CARGGSGG NLSYW	836	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-A02	Fzd7	GTFSSYAIS	295	GMIIPFLGIT NYA	521	CTRPYDAFD IW	1016	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-C02	Fzd8	YTFASYGM H	373	GWINAGNG NTTYA	558	CARLSVWK WEQVTNWF DPW	890	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-E02	Fzd8	GTFTSYAIS	305	GWINAGNG NTKYS	557	CTTGLFPYY RYNWWNDA FDIW	1022	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-A03	Fzd8	GTFSSYAIS	295	GWMNPNS GNTGYA	583	CAKWHIGAT GNWFDPW	729	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-H03	Fzd8	YTFTNNFMH	383	GGIFPIYGIS TYA	494	CARDRPTSS WYAFDYW	792	RASQGISNN LN	1072	DASTLQT	1193	CQQSFSAP ITF	1342
005S-F04	Fzd8	FSFSSTAMS	181	SYISSSGSI THYA	670	CARYGDYG DYW	954	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
005S-H04	Fzd8	YTFTNNFMH	383	GWINAGNG NTTYA	558	CARVATGNA FDIW	932	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-B05	Fzd8	FTFSSYWM H	239	AGISGSGKT TFYA	444	CARGGLLFD YW	831	QASQDISNY LN	1046	KASSLES	1226	CQQSYSTP RTF	1373
005S-F05	Fzd8	FTFTSSAVQ	251	GWMNPNS GNTGYA	563	CARRTAVAG TIDYW	914	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-G05	Fzd8	GTFSSYAIS	295	GWISPYNG NTNYA	573	CARGGWTN YGGNLDYW	841	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-H05	Fzd8	YTFTSYYMH	392	GRINPNSG GTNYA	537	CARVPDFW SGYLDYW	943	RASQGISRT LZ	1075	AASSLQS	1175	CQQTYSM PITF	1392
005S-D06	Fzd8	YTFTYRYLH	394	GGIIPIFGTA NYA	499	CARDSYPY GMDVW	800	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-F06	Fzd8	GTFSSYAIS	295	GRVIPILGV TNYA	545	CAREYLGSF DIW	815	RASQSVGSN LA	1108	GASSRAT	1213	CQQYGSS PPFTF	1409
005S-A07	Fzd9	FTFTGSAVQ	247	GGILPIYGT TKYA	509	CARGARLY GFDYW	822	RASQSVSRN LA	1114	GASTRAT	1217	CQQRSNW PITF	1335
005S-B07	Fzd9	FTFTSSAVQ	251	GWMNPNS GNTGYA	583	CARGRGQQ WLTGYYGM DVW	857	RASQGISSA LA	1076	GASTLQS	1215	CLQDYNYP FTF	1272
005S-C07	Fzd9	FTFSSYSMN	233	SYIENDGSI TTYA	654	CARAPYYYG SGSLFRLDY W	748	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-D07	Fzd9	GTFNSYAIA	291	GGIIPIFGTA NYA	499	CARAGSGY YNFDYW	740	RASQSINRW LA	1090	AASSLQS	1175	CQQTYNIPI TF	1386
005S-F07	Fzd9	FSFSSYGM H	182	AYINSRGSL MYYA	483	CAKTKLPIW	727	RASQSINRN YLG	1089	AASSRVT	1176	CQQYDSW PPTF	1402
005S-G07	Fzd9	GSFSGYAIN	274	GGIIPIFGTA NYA	499	CATGYYYDY YFDYW	972	RASQGISNN LN	1072	AASSLQS	1175	CQHYYNLP LTF	1309
005S-H07	Fzd9	GTFTNNFM H	303	GLVDPEDG ETIYA	520	CARTYRIVG ATPRYYYYG MDVW	931	RASQTINNQ LA	1125	KASNLET	1224	CQQANSFP VTF	1318
005S-B06	Fzd9	YIFTDYYMH	368	GWINPNSG GTIYA	562	CARGPRDS GYYPGGAF DIW	853	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
005S-D08	Fzd9	FAFSSHWM H	166	SAIDGSGG STYYA	592	CARDRQLG WAHWYFDL W	794	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-G08	Fzd9	YTFTGYYMH	379	GWINAGNG NTTYA	558	CARDRDYW	787	QTSQDINNN LN	1053	KASSLES	1226	CQQSYSSP PTF	1366
005S-C09	Fzd9	FTFSSYGMH	230	SAIGTGGG TYYA	595	CALLVGAAR GISYYYYYG MDVW	730	QASQDISNY LN	1046	AASTLQS	1179	CLQHKSFP TF	1276
005S-D09	Fzd9	YTFTSYAMH	389	GWINAGNG NTTYA	558	CARDRPYSS GWYYPAFDI W	793	RASQSVSSN QLA	1117	GASTRAT	1217	CQQRYNW PPSITF	1339
005S-E09	Fzd9	FNLRRYNM N	175	SRISNSGSL VYYA	627	CARDADSS GYYRYDAFD IW	762	RASQSVSSN LA	1116	DASNRAT	1188	CQQRNNW LYTF	1334
005S-A10	Fzd9	YTFTDYYMH	376	GIINPSGGS TSYA	513	CARHVYGS GTYNNWFD PW	878	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-D10	Fzd9	YTFTSYYMH	392	GVMSPNS ANTGYA	583	CARGGPIHY YYYYYMDV W	834	RASQGISNN LN	1072	AASTLQS	1179	CQQTNLFP YTF	1385
005S-H10	Fzd9	GAFSTSSIS	265	GRIIPVLGT ANYA	534	CAKGGWRS SFDPW	715	RASQSVSSN LA	1116	GASTRAT	1217	CQQYNSW PLTF	1421
005S-B11	Fzd9	YTFTSYDIN	390	GGFDPEDG ETIYA	492	CAKAGDWG LYGMDVW	695	RASQSISRW LA	1094	AASSLQS	1175	CQQTNTFP FTF	1386
005S-C11	Fzd9	FTFTGSAVQ	247	GGILPIYGT TKYA	509	CARGARLY GFDYW	822	RASQSVSRK LA	1113	GASTRAT	1217	CQQRSNW PITF	1335
005S-D11	Fzd9	YTFTNNFMH	383	GWINPNSG DTKFA	561	CAREANYDI LTGYIRPDA FDIW	806	RASQSLRSK LA	1106	GASTRAT	1217	CQQYANS PWTF	1401
005S-E11	Fzd9	GTFSSYAIS	295	GWINAGNG NTKYS	557	CTTTEYSSS PDYYYGMD VW	1025	QASQDISNY LN	1046	GASTLQS	1215	CQQLSRYP SLF	1331
005S-G11	Fzd9	GTFTRNSIS	304	GGIIPIFGTA NYA	499	CARSSDLRI FDYW	922	RASQSVSSN LA	1116	GASNRPT	1209	CQQYGSS PYTF	1413

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
005S-H11	Fzd10	YTFASYDIH	372	GWINAGNG NTTYA	558	CARDGIWDI FDYW	769	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-E12	Fzd10	YIFTDYYMH	368	GVIFPVYPT PDYA	551	CARGGSTG YYGMDVW	839	RASQSVGR WMA	1107	AASSLQS	1175	CQQANTFP FTF	1321
005S-F12	Fzd10	GTFSSYAIS	295	GRIVPIVDV VKYA	541	CARDTCSST SCSPDYW	801	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
006S-A01	Fzd10	FTFSSYSMN	233	SAIGTGGG TYYA	595	CAREGWFG ESPFGMDV W	810	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
006S-F01	Fzd10	YTFTRYAVH	385	GWISTFND NTNYA	576	CASPTGMTT NFDYW	959	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
006S-H01	Fzd10	YIFTDYYMH	368	GGIIPIFGTA NYA	499	CAKGSYYYD SSGYYWDA FDIW	723	RASQGISNN LN	1072	AASNLET	1166	CQQTSSTP LTF	1387
006S-A02	Fzd10	YIFTDYYMH	368	GGIIPLFGT TDYA	507	CARDITGAD GMDVW	775	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
006S-D02	Fzd10	GTFSSYAIS	295	GRIIPTVGT ANYA	533	CARDVCSG GSCSPDVW	802	RASQGISNN LN	1072	DASSLES	1189	CQQTYNTP RTF	1390
006S-E02	Fzd10	FTFTSSATQ	250	GGIIPIFGTA NYA	499	CARDGSSG WYSPNAFDI W	770	RASQGISNN LN	1072	AASSLQS	1175	CLQHNGYP ITF	1277
006S-H02	Fzd10	FTFRMYGM H	200	SRISPDGRT TTYA	628	CARSPRWY DAFDIW	920	RSSQSLLHS NGYNYLD	1138	RVSSRFS	1251	CMQGTHW PPTF	1288
006S-A03	Fzd10	YIFTDYYMH	368	GWINAGNG NTTYA	558	CARDPIMFG DQPGWFDP W	784	RASESVSSN LA	1055	GASSRAT	1213	CQQYNKS PSF	1419
006S-B03	Fzd10	GTFSSYAIS	295	GWINAGNG NTKYA	556	CAREGYDF WSGPYAFDI W	811	RASQTISRYL N	1126	EVSSLQG	1203	CQQSYSTP WTF	1376
006S-C03	Fzd10	GTFSSNVIS	293	GGIIPIFGTA NYA	499	CARGGYYY GMDVW	843	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-B01	Fzd1	YIFTDYYMH	368	GGIIPIFGTA NYA	499	CARMSSDY YDSSGYYR RGMDVW	895	RASQGISNN LN	1072	GASTLQS	1215	CQQADSFP PTF	1312

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
014S-D01	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	RASQSISSH ZN	1096	AASSLQS	1175	CQQSYSTP LTF	1372
014S-E01	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	RASQSIZZYZ N	1105	AASSLQS	1175	CQQSYSTP LTF	1372
014S-G01	Fzd4	GTFSSYAIS	295	GWMNPNN GNTTYA	581	CARHYYGS GNYRDW	879	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP FTF	1368
014S-A02	Fzd4	FTFSSNAMH	223	SGISGSGG STYYA	608	CAKPGIAAA GTNNWFDP W	725	RASQGISSA LA	1076	GASTVES	1218	CQQSYSTP RTF	1373
014S-B02	Fzd4	FTFSSYAMH	227	SGISGSGS STYYA	611	CARPSTTSF GMDVW	902	RASQSVSSN LA	1116	GASTRAT	1217	CQQYDTPL RTF	1403
014S-C02	Fzd5	YTFTSYYMH	392	GRINPNSG GTNYA	537	CARVPDFW SGYLDYW	943	RASQGISSA LA	1076	AASSLQS	1175	CQQTYSM PITF	1392
014S-D02	Fzd5	GTFSTYAIS	299	GIINPSGGS TSYA	513	CARAKGSG WYVGSAFDI W	744	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-E02	Fzd5	FTFSDSYMS	206	GFIRSKAYG GTTEYA	490	CARATQELL LPYGMDVW	758	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-F02	Fzd5	YTFTSYYMH	392	GRINPNSG GTNYA	537	CARVPDFW SGYLDYW	943	RASQGVSTZ LS	1079	AASSLQS	1175	CQQTYSM PITF	1392
014S-G02	Fzd6	YTFTSYYMN	392	GIISPSGGS TSYA	516	CARWGDYG DLYYFDYW	951	RASQGISSA LA	1076	ATSTLQS	1183	CQQVNSY PPTF	1399
014S-H02	Fzd6	YIFTDYYMH	368	GRINPNSG GTNYA	537	CARARSSG WTDAFDIW	751	RASQSVSS WLA	1120	AASTLQT	1180	CQQSYSTP TF	1376
014S-A03	Fzd6	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-B03	Fzd6	FTFSSYNMN	232	GRIKSKAN GGTTDYA	535	CARAGDSP DYW	739	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-E03	Fzd8	GTFSSYAIS	295	GWISPYNG YTKYA	574	CARAMWSY GQQNAFDI W	745	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-G03	Fzd8	FTFTSSAVQ	251	GWMNPNS GNTGYA	583	CARRTAVAG TIDYW	914	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
014S-H03	Fzd8	YTFTSSAIH	387	GRINPNSG GTNYA	537	CARVKWEL AIDYW	940	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-B04	Fzd8	YIFTDYYMH	368	GWMNPNS GNTGYA	583	CARGGSRY DFWSGHWY FDLW	837	RASQGISNY LA	1074	AASSLQS	1175	CQQSYSTP FTF	1368
014S-E04	Fzd8	YTFTGYYMH	379	GRINPNSG GTNYA	537	CARDVPKLV TRGVAYGM DVW	804	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-F04	Fzd8	YSFTTYGMN	371	GWINAGNG NTTYA	558	CARAAAGSY GGGYW	736	RASQGISNN LN	1072	EASSVAS	1197	CQQSYTST PLNSF	1381
014S-G04	Fzd8	FTFSSYGMS	231	SAISGSGG STYYA	600	CARDLTPFT QQQLVLGLL	780	RASQSVSGY LA	1112	GASTRAA	1216	CQQYNYW PPAF	1423
014S-H04	Fzd8	FTFTSSAVQ	251	GRIVPAIGF TQYA	540	CARSGYNR RGYFDYW	919	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-A05	Fzd8	GTFSSYAIS	295	GGIIPIFGTA NYA	499	CARVTLGAS VDAFDIW	949	RASQGISNN LN	1072	DASSLES	1189	CLQHNSLP FTF	1278
014S-B05	Fzd8	GTFSSYAIS	295	GWVSPNTG NTVYA	587	CTTDRRYST YFDLW	1021	RASQSVSSN LA	1116	GVSNRAT	1223	CQQYNIWP RTF	1418
014S-C05	Fzd8	YTFASYGM H	373	GWINAGNG NTTYA	558	CARLSVWK WEQVTNWF DPW	890	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-D05	Fzd8	GTFTSYAIS	305	GWINAGNG NTKYS	557	CTTGLFPYY RYNWWNDA FDIW	1022	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-F05	Fzd9	FTFTGSAVQ	247	GGILPIYGT TKYA	509	CARGARLY GCDYW	821	RASQZVSRZ ZA	1127	GASTRAT	1217	CQQRSNW PITF	1335
014S-G05	Fzd9	FTFSSSWM H	226	SAIGTGGG TYYA	595	CARKVKGY CSGGSCYG YW	884	RVSQGISSA LA	1151	AASSLQS	1175	CQQTFSVP WTF	1384
014S-H05	Fzd9	FTFSNYAMT	216	STISGSGVS TFYA	648	CARHGRIAA DIW	876	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-A06	Fzd9	FTFZZSZVQ	254	GGILPIYGT TKYA	509	CARGARLY GFDYW	822	RASQSVSRN LA	1114	GASTRAT	1217	CQQRSNW PITF	1335

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
014S-B06	Fzd9	FTFSSYSMN	233	SYIENDGSI TTYA	654	CARAPYYYG SGSLFRLDY W	748	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-C06	Fzd10	FTFTGSAVQ	247	GGILPIYGT TKYA	509	CARGARLY GFDYW	822	RASQSVSRN LA	1114	GASTRAT	1217	CQQRSNW PITF	1335
014S-D06	Fzd10	FTFSRYAMH	219	SGIGVGGG TYYA	605	CARDAYNW FDPR	763	RASQSISRY LN	1095	AASSLQS	1175	CQQRYSTP LTF	1340
014S-F06	Fzd10	YIFTDYYMH	368	GVIFPVYPT PDYA	551	CARGGSTG YYGMDVW	839	RASQSVGR WMA	1107	AASSLQS	1175	CQQANTFP FTF	1321
014S-G06	Fzd10	FTFSSYAMH	227	SAIGAGGG TYYA	593	CARDAYNW FDPW	764	RASQSISSYL N	1096	AASSLQS	1175	CQQSYSTP LTF	1372
014S-H06	Fzd10	FTFSSYDMN	229	SAIGTGGG TYYA	595	CARDAYNW FDPW	764	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-A07	Fzd10	FTFSNAQMS	210	SAIGTGGG TYYA	595	CAREGSYY DWYFDLW	809	RASQNIGSR LA	1080	GASNRAS	1208	CQQYNHW PPLFTF	1417
017S-E08	Fzd8	IIFSPNDMG	313	ALISSGGST SYA	450	CHFGVASV GLNYW	980						
017S-H08	Fzd8	RTFSSFVMG	346	AAVSASGG YTWYA	432	CNLAQRGET YW	998						
017S-A09	Fzd8	LAFNGYTM G	317	AAISWSDN TYYA	414	CAAGFPTVF VVDGEYDY W	660						
017S-B09	Fzd8	FTLDYYAIS	255	ADITSGGST NYA	437	CNAVTYNGY TIW	994						
017S-C09	Fzd8	LTFSDYTVG	319	ASSTGGGV FENYA	464	CNAVTYNGY TIW	994						
018S-D06	Fzd4	RIFSSYAQA	330	PRIPSDSTT FYA	590	CEVHNFGAT YW	979						
018S-E06	Fzd4	RTFSNYVM G	344	AVISRSGG NTYYT	472	CNAVSTDW TTDYW	992						
018S-F06	Fzd4	RTFSTYGM G	349	AAISWSDN TYYA	414	CNSFPLRLH DW	1002						
018S-G06	Fzd5	LAIDDYYMV	318	SYISTSDGS TYYA	673	CNAVTYNGY SIW	993						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
018S-H06	Fz85	LAFNGYTM G	317	AQISWTGG STDYA	460	CNADYGTW YGIGW	985						
018S-A07	Fz85	LAFNGYTM G	317	AAISWMSN TYYA	412	CNMGLGYS EYRPLGYW	999						
018S-B07	Fz85	SAFSNYAM G	355	AAITWSGA RTYYA	426	CNAVWKFG TTHW	995						
018S-C07	Fz87	LTIDDYYVV	323	SYISAGDGF TYYA	656	CNAVTYNGY TIW	994						
017S-F09	Fz84	GSFSGYYW S	277	GEINHSGST NYN	489	CARDLRFYS SSWRRVGM DVW	778	RSSRSLLHT SGYNYLD	1147	LGSNRAS	1237	CMQGTRW PTF	1290
017S-G09	Fz84	YTITTYAIH	396	GWINADTG DTAYS	555	CARGWTTIS SLGVW	872	RSSRSLLHT NGYNYLD	1146	LGSNRAS	1237	CMQALQTP LTF	1281
017S-H09	Fz85	NIFRIYAIA	326	AALTGQRT TNYA	427	CNTVTYNAG CYKKYW	1004						
017S-A10	Fz85	LAFNGYTM G	317	ASITWNGR YTYYA	462	CNARLDAVY GHSRYDSW	968						
017S-B10	Fz85	NFFSNYPLG	325	GAISRTGS GTFYA	484	CAAGVTGS WRYW	664						
017S-C10	Fz88	RSFSNYRVA	335	AVSWSVGM TYYA	479	CNAVTYNGY TIW	994						
017S-D10	Fz88	GTFGSYAV G	288	GLISRNAGN TLYA	518	CNAVNGRL NYW	991						
017S-E10	Fz88	RTFSSYSLA	348	AAVSASGA NTYYA	431	CAAPQSPN MYIRTDQLW WYKYW	687						
018S-D07	Fz81	RSFSTYPM G	336	TVISGSGGS TYYA	675	CAAGPTLPF RYW	683						
018S-E07	Fz81	RAFSNYAM G	328	AAINWSGD SAYYA	406	CNARLSFAG GMQYW	989						
018S-F07	Fz81	IKSMFDMNF MG	314	AFITRGGTT RYG	438	CNAVSTDW TRDYW	992						
018S-G07	Fz81	LTIDDYYMV	322	SYIGTSDGT TYYA	655	CNAVTYNGY TIW	994						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
018S-H07	Fzd4	RVFSSYAQA	354	AGIASDSTT FYA	439	CKVHNFGAT YW	983						
018S-A08	Fzd4	RIFSSYAQA	330	ASIPSDGTT FYA	461	CKVHNFEAT YW	982						
018S-B08	Fzd4	LTFSTYGMG	321	AAINWSGR STVYA	407	CNSFPLRLH DW	1002						
018S-C08	Fzd4	RTLSSYVVG	351	ALISLSGAS TYYA	449	CNAVSTDW TTDYW	992						
018S-D08	Fzd5	IKSMFDMNF MG	314	AFITRGGTT RYG	438	CNAVSTDW TRDYW	992						
018S-E08	Fzd5	RTDGMQAM G	338	GAITWSLG SAFYA	486	CNVLAQND GDYRTYG	1007						
018S-F08	Fzd5	RTFSSFVMG	346	AAVSASGG YTWYA	432	CNAVWKFG TTHW	995						
018S-G08	Fzd5	RTFSSFVMG	346	AAVSASGG YTWYA	432	CNAVCKFGT THW	990						
018S-H08	Fzd5	RTFSSFVMG	346	AAVTASGG YAWYA	434	CNAVWKFG TTHW	995						
018S-A09	Fzd8	ITFSFNSVG	316	AVFIAGYGA YYA	470	CNGVTYNG YTIW	997						
018S-B09	Fzd8	HDFSSTYGV G	310	ATISWGGT NIA	468	CAAQKPYYN GHFYADDK HYDHW	688						
018S-C09	Fzd8	ITFGFDSVG	315	AVFNAGYR AYYA	471	CNAVTYNGY TIW	994						
018S-D09	Fzd8	RTFSWYSM G	350	AAVSWSGV STYYP	433	CNAVTYNGY TIW	994						
018S-E09	Fzd8	ITFSFNSVG	316	AVFIAGYGA YYA	470	CIGVTYNGY TIG	981						
018S-F09	Fzd8	RTDGMQAM G	338	GAITWSLGI AFYA	485	CNVLAQND GDYRTYW	1008						
018S-G09	Fzd8	HDFSSTYGV G	311	AAISWRGT NIA	413	CAAQKPYYN GHFYADDK HYDHW	688						

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
021S-A01	Fzd8	DSVSSNSAA WN	160	GRAYYKSR WYYDYA	524	CVRDLRPSG DLNFDYW	1029	RASQSIGSS LH	1037	YASQSVS	1260	CHQSGRV PVTF	1268
021S-C01	Fzd1	GSISSGGYS WS	283	GSIYHSGST YYN	547	CARFYYDIL NGYSYFDY W	817	RSSRSLLDT DDGNTYLD	1142	TLSHRAS	1259	CMQSIQLP WTF	1295
021S-D01	Fzd1	FTFSSYGMH	230	AVISYDGSN KYYA	475	CAKGSVFGL KAGGYADY W	721	RSSQSLVHS DGNTYLS	1140	KISNRFS	1230	CMQATQFP HTF	1283
021S-E02	Fzd8	YTFTSYGIS	391	GWISAYNG NTNYA	570	CARDGTPFY SGSYYGSW	772	QGDSLRTYY AS	1052	GKNNRPS	1219	CNSRDNS GKHKVF	1300
021S-G02	Fzd8	DSVSSNSG AWN	162	GRTYYRSK YYNGYA	544	PRLDYW	1034	RSSQSLLDS DDGNTYLD	1133	MLSSRAP	1240	CMQRLEFP YTF	1294
021S-A03	Fzd8	DSVSSNSAA WN	160	GRTYYRSK WYNDYA	542	CARSQATG ERFDYW	921	RSSQNIFQS LN	1131	SASSLQS	1254	CQQSYNS PITF	1349
022S-H06	Fzd4	FTFSSYAMS	228	SVISTSGGT VLYT	652	CADGSGTS HR	690	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
022S-A11	Fzd10	YIFTDYYMH	368	GGIFPIFGT ANYA	493	CAKGSYYYD NSGYYWDA FDIW	722	RASQGISNNI N	1071	AASNLET	1166	CQQTYSIP FTF	1391
OMP-18R5		GFTFSHYTL S	270	VISGDGSYT YYADSVKG	677	NFIKYVFAN	1033	SGDKLGKKY AS or SGDNIGSFY VH	1152 or 1153	EKDNRPS G or DKSNRPS G	1200 or 1201	SSFAGNSL E or QSYANTLS L	1435 or 1436
027S-H02	Fzd5	FTFSSYAMS	228	SAISGSGG STYYA	600	CAKGLWGP LLNW	718	RASQSVSSN YLS	1118	GASSRAP	1212	CQQRTNW PPRVTF	1337
027S-B03	Fzd8	DSVSSNSAT WN	161	GRTYYRSK WYSDYA	543	CTRGNWNV GLANW	1014	SGTSSNIGA GYDVH	1157	GNNNRPS	1219	CSAWDDN LNGVVF	1432
027S-E01	Fzd5	RSFSIYNTA	334	AAISWSGG STYYA	418	CNVITIVRG MGPRAYW	1066						
004S-D05	Fzd5	LTFSIYAMH	320	SAISGDGAL TYYA	597	CARGVYPYS SKHKPSYYY YGMDVW	870	QASQDISNY LN	1046	AASSLQS	1175	CQQSYSTP LFTF	1371
004S-D04	Fzd5	YDFTTYGIH	367	GGVIPAFGA TDYS	511	CARGYYYG MDVW	874	RASQSISSYL N	1096	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
004S-B05	Fzd5	GTFSSYAIS	295	GWINAGNG NTTYA	558	CASGLGYFD YW	957	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-G03	Fzd5	YTFTNNFMH	383	GGIIPIFGTP HYA	502	CARTLTTPP YYYGMDVW	929	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F03	Fzd5	FTFSNSDMN	214	SAIGTGGDT YYA	594	CTRDLYGGY RDYW	1013	KSSQSLLHS DGYTYLY	1036	LGSNRAS	1237	CMQGLQT PWTF	1286
004S-C04	Fzd5	YIFTGYYMH	369	GRINPNSG GTNYA	537	CARGGEYS SGWTYYYY YGMDVW	827	RATQTISTYL N	1129	AASRLQS	1171	CQQYYSYP WTS	1427
004S-B06	Fzd5	YTFTYRYLH	394	GMINPIGGS INYA	522	CARDVMDV W	803	RASQGISNN LN	1072	AASALQS	1165	CQHLNNFP LTF	1303
004S-F06	Fzd5	FSVGSNYM T	186	SSISSGNSY IYYA	634	CARGPKTM WEDRPDYW	851	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-A04	Fzd5	FTFSTYSMI	245	GFIRSKDYG GTTEYA	491	CARLTGGAV AGTHRDYW	892	RASQGISNN LN	1072	GASSLQS	1211	CQQSHSS PRTF	1346
004S-A05	Fzd5	FTFSSYVMS	237	SAIGTGGG TYYA	595	CARGSSGY YVAW	862	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F05	Fzd5	FTFSNHYMS	212	AGVSIDANK KYYA	447	CARDQNDS WYRSDYW	785	RSSQSLLHS DGYTYLY	1135	LGSHRAS	1235	CMQGLQT PHTF	1285
003S-C01	Fzd1	GTFSSYAIS	295	GRINPNSG GTNYA	537	CARGSGYD FFDYGMDV W	861	RASQSISNN LN	1092	AASSLQS	1175	CQQSYNTP FTF	1350
003S-H01	Fzd1	DTFSNYVLS	164	GLVDPEDG ETIYA	520	CAKASTPMV QGAPDYW	697	RASQSIGSN LD	1085	AASTLQS	1179	CQQNYATP RTF	1333
003S-H02	Fzd1	GTFNRYAIT	289	GGIIPIFGTA NYA	499	CATTQGVYS SSWYGGGR AFDIW	976	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-H04	Fzd1	YTFTYRYLH	394	GRINPNSG GTNYA	537	CWGSGSYYG DYW	1030	RASQGISNN LN	1072	AASSLQS	1175	CQQANSFP ITF	1316
003S-A05	Fzd2	FTFSSYAMH	227	SSISWNSG RVDYA	638	CARGSGIAA SGSYW	860	TSSQSLLHS DGKTYLY	1162	LGSNRAS	1237	CMQGTHW PYTF	1289
003S-B05	Fzd2	FTFSNAWM S	211	STIAGSGG RTYYS	643	CAKDSIGRR GRGAPQPY YYYGMDVW	709	KSSQSLLHS DGKTYLY	1036	LGSNRAS	1237	CMQSLQS PLTF	1296

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
003S-F05	Fzd2	FSFSTYTMS	184	SRINGDGS STRYA	624	CARAIVGAT GLNRFKAFD IW	743	KSSQSLLHS DGKTYLY	1036	LGSNRAS	1237	CMQNTHW PLTR	1293
003S-G05	Fzd2	STFTNAWM S	363	SAIGTGGG TYYA	595	CARDRVTLR GGYSYGTD AFDIW	796	RSSRSLLHS NGNTYLR	1145	LASRRAS	1231	CIQNTHWP LTR	1270
003S-H05	Fzd2	FTLSTYNMN	257	SRINYDGSA TTYA	626	CARDRDIVV VPAQRGEG GFDPW	786	KSSQSLLHS DGKTYLY	1036	MGSYRAS	1239	CMQGTHW PLTF	1287
003S-A07	Fzd2	FTFSSYAMS	228	SAISGSGG STYYA	600	CAKGGRDG YKGYFDYW	714	KSSQSLLHS DGKTYLY	1036	LGSNRAS	1237	CMQNTHW PLTL	1292
003S-C07	Fzd2	FSFRSYSMS	178	SAIGTGGG TYYA	595	CTTTTVTTS W	1026	RTSQSVSSN LA	1150	DASNRAS	1187	CQQYGSS PYNF	1412
003S-F07	Fzd2	FSFSSYGM S	183	SHISSGGAT IDYA	619	CARDGGYW	766	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-G07	Fzd2	FTFSSYWM H	239	SYISGDSGY TNYA	658	CARDNGYC SGGSCYATY YGMDVR	783	RASQAISSYL A	1060	KASTLDT	1228	CQQADTFP FTF	1313
003S-B08	Fzd2	FTFSSZZMH	240	AVISYDGSN RZYA	476	CARSYYDSS GYPRKDAFD IW	927	KSSQSLLHS DGKTYLY	1036	LGSNRAS	1237	CMQTLKAP LTF	1299
003S-F08	Fzd2	ZSVSSNYM S	401	SRINSDGST ISYA	625	CARARLLGG YYTPDRMD VW	750	RSSQYLSSA YLA	1141	GASSRAT	1213	CQQYGSS PTF	1411
003S-H08	Fzd2	FTFNRHALS	197	ALISSNGDH KYYT	451	CARDLMVG RNKLDYW	777	QASQGISNN LN	1049	AASSLQS	1175	CQQSYSTP AFTF	1367
003S-A09	Fzd2	FTFSSSNMN	225	SGISGSGS STYYA	611	CARGRVWS SRDYW	859	RSSQSLLHS DGYTYLY	1135	LGSNRAS	1237	CMQGTHW PLTF	1287
003S-B09	Fzd2	FNIRRZNMZ	174	SAIGTGGG TYYA	595	CARGDSGS YRDYW	823	RSSESLLHS DGKTYLY	1130	LGSNRAS	1237	CTQTVQFP ITF	1433
003S-C09	Fzd2	FTFSSSAMH	224	SGISGSGTT TYYR	612	CARRLAVA GAEFDPW	912	RASQGISNN LN	1072	SASNLQS	1252	CQQSYSTP WTF	1378
003S-F09	Fzd4	FTFSNSDMN	214	GRIKSKAYG GTTEYA	536	CARQYYFDY W	909	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
003S-H09	Fzd4	FTFSSFGMH	221	SVISSGGSP YYA	651	CATASGDFD YW	968	RASQSIGSN LN	1086	RASTLES	1248	CQQTYTTP RF	1398
003S-A10	Fzd4	FTFDDYAMH	191	AIVSYDGTY KYYS	448	CARQTRGG TTDGW	907	RASQGISNN LN	1072	YASSLQS	1262	CQQSHSP PGTF	1345
003S-B10	Fzd4	FTFSSHSTH	222	SAISASGDS TFYA	596	CARPIVGAT AFDIW	900	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-G10	Fzd4	FTZSSYSMN	264	SYSSGNSG YTNYA	674	CARGVVGS GAFDIW	868	RASQSIVSYL N	1104	DASNLQS	1186	CQQGYSA PWTF	1329
003S-B11	Fzd4	FTFSDYYMS	208	SAIDGAGRT YYT	591	CARAIPGDY DYW	742	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-C11	Fzd4	FTFTSYAMH	252	GGIIPIFGIA NYA	496	CARTGRGY YGMDVW	928	RASQSIGSN LD	1085	AASTLQS	1179	CQQSYSTP RTF	1373
003S-D11	Fzd4	FTFSSYSMS	234	SYISGDSGY TNYA	658	CARAGVATI AFDYW	741	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-F11	Fzd4	FTFDDYGM H	192	SAISGSGG STYYA	600	CTTPNYYDS R	1023	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
003S-E12	Fzd4	GTFSSYAIS	295	GWINAGNG NTTYA	558	CARHYYGS GSYPDW	880	ZPZQTZZSH LN	1164	PASSLQS	1242	CQQSYSTP LTF	1372
004S-A01	Fzd4	FTFSTYGMH	244	SYISSSSAI YYA	671	CARGGLDG PIDYR	830	RASQGISNN LN	1072	AASTLQS	1179	CQQGNNF PFTF	1326
004S-G01	Fzd4	FTVSSHSM G	260	SLVSFDGS KEHYA	621	CARLGSTPD YW	887	RASQGISNN LN	1072	AASSLQS	1175	CQQYYTYP YTF	1428
004S-C02	Fzd4	FTFSSYGMH	230	AVISYDGSN KYYA	475	CASDPVTAA TR	956	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-D02	Fzd4	FSFSSYGM S	183	SGISGSGR STYYA	610	CAKDGYW	699	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-A03	Fzd4	FTFSSYAMH	227	SGINWNGG STGYA	606	CARPAGSA QNWFDPW	899	RASQGISNN LN	1072	DASNLET	1185	CHQSYSIP RTF	1269
004S-B03	Fzd4	FSFSRYGM S	180	SGVGGSGG STZYA	617	CARDGSW	771	RASQDVDT WLA	1066	DASTLET	1191	CQQGYNIP WTF	1328
004S-C03	Fzd4	YTFTSYAIS	386	GIINPSGGS TSYA	513	CARQIGWEL MPDIW	906	QASQDISSY LN	1047	AASTLQS	1179	CQQAISFP LTF	1315

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
004S-C05	Fzd5	ZZZTDYYZQ	403	GGMNZNR GNTGYA	510	CANGSYAQ HLW	732	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-G05	Fzd5	FTFSSYWM H	239	STISPSGLYI YQA	649	CAKDKVPYS YGPNFDYW	703	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-E06	Fzd5	FFFSGYWM S	169	ANIKQDGS EKYV	459	CARVFPLHD YW	934	QASQDISNY LN	1046	KASSLES	1226	CQQANSFP YTF	1319
004S-C06	Fzd5	FPFSTFSMN	176	AGISWNSG TIDYA	446	CARSGPAA MVYYYYGM DVW	918	RSSQNVSSY LA	1132	GASTRAT	1217	CQHRANW PQTF	1305
004S-E07	Fzd6	FTLSSHHMN	256	SAIGTGGG TYYA	595	CAAPDYW	686	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F07	Fzd6	FSFSKKYMT	179	SSIDGNGD HVFYA	629	CARPYYYDS SGYDPMGD YW	904	QASQDITNY LN	1048	KASTLES	1229	CQQSYSAP YTF	1356
004S-C08	Fzd6	FTVSSNYM N	261	SAIGTGGG TYYA	595	CAQGTYW	735	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-F03	Fzd6	FTFDDYYMN	193	SAVSGNGG GTFYA	604	CARGGNYG SGDYW	833	RASQSISZW LA	1102	EASTLQS	1198	CQQTYTPP FTF	1397
004S-G09	Fzd6	GTLNNHTLS	308	GRIIPIFGTA NYA	526	CARDRRGY GMDVW	795	RASQAISNS LA	1058	DASNLET	1185	CQQAYSFP WTF	1324
004S-B10	Fzd6	FTFSDYYMS	208	SGINWNSA KIGYV	607	CARIGAGGA FDIW	881	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-H10	Fzd6	FIFSDYYMS	170	AVITSGGTF KYYA	477	CARNGIAAA EDYW	896	RASQSISTYL S	1101	GASSLES	1210	CQQSYSPP FTF	1362
004S-B11	Fzd6	FTFSSSWM H	226	SGISWNSG SIGYA	615	CARYSSGG SLDYW	955	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-D11	Fzd6	YZFZZZYMH	400	GRINPNSG GTNYA	537	CARARSSG WTDAFDIW	751	RASQSVSS WLA	1120	AASTLQT	1180	CQQSYSTP TF	1376
004S-F11	Fzd6	FTFSSYAMS	228	SSISGGGR HTYYA	632	CARPYSSSR QGDYW	903	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
004S-G11	Fzd6	YIFTDYYMH	368	GWINPNSG GTNYA	564	CARDRPGF DPW	790	RASQSVSSY LA	1121	GASSRAT	1213	CQQYAISY TF	1400
004S-B12	Fzd6	FTFSSYWIH	238	SYISGDSGY TNYA	658	CAKGIRWFD PW	716	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
004S-C12	Fzd6	YIFTDYYMH	368	GWMNPNS GNTGYA	583	CASSHYAPG MDVW	962	RASQGISSY LA	1076	RTSTLES	1250	CQQSYSTP WTF	1378
004S-F12	Fzd7	FTVGNNYM S	258	SSITTTSTL YA	640	CARGKEGR YSNYEAAW	844	RASQSISSYL N	1098	AASTLQT	1180	CQQSYSIP FTF	1358
005S-B01	Fzd7	FTFRSYGM H	202	SLISGSGDN TNYA	620	CARREPLYS SRRGAFDIW	910	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-C01	Fzd7	FTFSSYSMS	234	SAISGSGG STYYA	600	CTRTIVGAT PHYW	1017	RASQGISNN LN	1072	KASSLQS	1227	CQQSYSLP YTF	1360
005S-F01	Fzd7	FTVSSNYMS	262	SAISGSGAT TTYA	598	CAKGAGYG SGSWQAAW	711	RASQSVSSS YLS	1119	GASSRAT	1213	CQQRYKS YTF	1338
005S-B02	Fzd8	YSFTNYAM H	370	GRIIPIFGTA ZYA	527	CARGTFLE WLLTNYGM DVW	866	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-D02	Fzd8	GTFSSYVIS	297	GWIGPHNG NTNYA	554	CATGWPRY YYGMDVW	971	RASQSVSSN LA	1116	NTSNRAT	1241	CQHYNNW PFTF	1308
005S-G02	Fzd8	YTFTSYYMH	392	GGIIPIFGTA ZYA	500	CARLPYYDF WSGYYGGR TGFDYW	889	RASQSVSTN LA	1122	DASNRAT	1188	CQQRSNW PPQITF	1336
005S-H02	Fzd8	YTFTYRYLH	394	GWINAGNG NTTYA	558	CARASLYYD YVWGSYRH YYFDYW	753	QASQDISHY LN	1044	AASSLQS	1175	CQQSYSTP LTF	1372
005S-B03	Fzd8	GTFSSYAIS	295	GIINPSGGR TTYA	512	CATSFGGG WIVVDTSLW YW	975	RASQSINSN LA	1091	GASSRAT	1213	CQQYGSS PYTF	1413
005S-C03	Fzd8	GSFSGYAIS	275	GGIIPIFGTA NYA	499	CRVDAFDIW	1009	RASQSVSSS YLS	1119	DTSNRAT	1194	CQQYGSS PITF	1408
005S-E03	Fzd8	FTFTSSAVQ	251	GGIIPIFGTA NYA	499	CARSSGWQ NRFAFDIW	923	RTSQSISSYL N	1149	AASTSQS	1181	CQQSFSS WTF	1343
005S-F03	Fzd8	YTFTYRYLH	394	GWINAGNG NTKYS	557	CATDLPVRK GFTYYDILT GSYGMDVW	970	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-B04	Fzd8	YTFTNNFMH	383	GGIIPIFGTA NHA	498	CARGLRYFD WPQGIYYYY GMDVW	848	QASHDINIAL N	1039	AASSLQS	1175	CQQSYSSP LTF	1365

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
005S-C04	Fzd8	YTFTSYYMH	392	GRINPNSG GTNYA	537	CARGGLLFD YW	831	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-D04	Fzd8	FTFSTYSMS	246	STIGTGGGT YYA	645	CARVGWLR FLDYW	938	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-G04	Fzd8	GTFSSYAIS	295	GWMSPSS GNAGYA	585	CARNNFLRA FDIW	897	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-A05	Fzd8	FAFSSYAMS	167	SRIDTDGST TVYA	622	CARAPSYSS GWYVRW	747	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-C05	Fzd8	YTFTYYAMH	395	GIINPSGGS TSYA	513	CARELLFMT TVTSPFIW	812	RASQGISNN LN	1072	RASSLQS	1247	CQQANSY PLTF	1320
005S-E05	Fzd8	GTFSSYAIS	295	GGIIPIFGTA NYA	499	CAIRAFDIW	694	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-C06	Fzd8	ZTFSZYDMH	402	SSISSSSHY KYYA	635	CARVRSKAV AGTLPKRLF DIW	947	RASQSVSSS YLS	1119	AASRRAT	1172	CQQYSNW PFTF	1424
005S-E06	Fzd8	YTFTSYYMH	392	GWMNPNS GNTGYA	583	CARGNPTS GHWWPAA TFSDYW	850	QASQDISNR LN	1045	SASRLQI	1253	CQQSYRTP RTF	1354
005S-G06	Fzd8	GTFSZZTIS	302	GWMNPDS GKTGYA	579	CARWAFPIP NAFDIW	950	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-H06	Fzd8	YTFTNNFMH	383	GGIFPIYGIS TYA	494	CARDRPSSS WYAFDYW	791	RASQGISNN LN	1072	DASTLQT	1193	CQQSFSAP ITF	1342
005S-A08	Fzd9	GTFSZYAIS	301	GGIIPIFGTA NYA	499	CARGGLLRF GDGWGMG MDVW	832	RASQSISSK SLA	1097	GASTRAT	1217	CQQYGIAP TF	1407
005S-C08	Fzd9	YTFTDYHMH	375	GWINAGNG NTTYA	558	CARASSWY LHYYYGMD VW	755	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-E08	Fzd9	FIFSZYAMS	171	SSISAAGAY KYYA	631	CARRGYSS GWRDAFDI W	911	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-F08	Fzd9	YTFTSYYMH	392	GWINAGNG NTTYA	558	CAKDVNYW	710	QASQGISNY LN	1050	AASSLQS	1175	CQQTYSTP TTF	1395

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
005S-H08	Fzd9	GTFSSYAIS	295	GRIIPILGTP NYA	531	CARDRLAFD YW	789	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-A09	Fzd9	FAFSSHWM H	166	SAISVSGGT TFYA	602	CARWGKRL RGSPYYFDY W	952	RASQSIGSN LD	1085	RASTLQS	1249	CQQSYSTP SF	1375
005S-F09	Fzd9	FTFSIYGMH	209	SGISWNSG NIGYA	614	CARGPLPTK IGGHYMDV W	852	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-B10	Fzd9	FTFSTXWM S	241	AVMYSGGT TYYA	478	CARLSYYYD SSGPKGDAF DIW	891	RASQGISNN LN	1072	AASSLQS	1175	CQQGNNF PLTF	1327
005S-C10	Fzd9	FSLSSYGM H	185	SSISSSSSYI YYA	636	CARSGMVK WLRSFDYW	917	RASQDIGSF LA	1061	AASSLQS	1175	CQKYNRAP FTF	1310
005S-E10	Fzd9	FTFTSSAMQ	249	GVINPGSG GTSYN	552	CARGYGDY VWGENYFD YW	873	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-F10	Fzd9	YTLSNYGIS	397	GWISAYNG DTKYA	568	CARFDYFG GMDVW	816	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-G10	Fzd9	YTFTRYAVH	365	GGIIPFFNT VNYA	495	CAADRSPYY YDSSGYYPD AFDIW	679	RASQGISNN LN	1072	QASSLDS	1244	CQQSYNV PYTF	1352
005S-A11	Fzd9	FTFSSYDMN	229	SGISWNSG YIGYA	615	CAKGSLLLG YYGMDVW	720	RASQSISNN LN	1092	DASTLKR	1192	CQQSYNTP RTF	1350
005S-B12	Fzd10	FTZSSYDMH	263	SSISGLGGS TYZA	633	CAREAGTTG GWFDPW	805	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
005S-D12	Fzd10	FTFSDHYMD	204	STIGPAGDT YYP	644	CARASTSGD YSLW	756	RASQSVSTS YLA	1123	GASTRAT	1217	CQQYGAS PWTF	1406
006S-B01	Fzd10	YTFTNYCTR	384	GLVCPSDG STSYA	519	CARRTSASD IW	915	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
006S-C01	Fzd10	FTFTZSAVQ	253	GGFDPEDG ETIYA	492	CTTDPLELP WYW	1020	RASQGISSA LA	1076	SASNLQS	1252	CQQAISFP LTF	1315
006S-E01	Fzd10	YTFTGYYMH	379	GIINPSSGR TDYA	514	CARDLTYYY DSSGHSPLG AFDIW	781	RASQSVTSS LA	1124	GASTRAT	1217	CQQYNDW PPTF	1416

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
006S-G01	Fzd10	FTFSDFGMN	203	AGISGGGG STDYA	443	CARDSDFW YYYGMDVW	797	KSSQSVLYS SNNKNYLA	1037	STNTRSS	1257	CQHRNFF	1306
006S-B02	Fzd10	VSFSGYAM H	366	AYINSGSSE MNYA	482	CAREEWELF GMDVW	807	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
006S-G02	Fzd10	YTVTSYAMH	399	GGIIPIFGTA KYA	497	CAKGGQWL YGMDVW	713	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-A01	Fzd1	YTFTSYYMH	392	GWVSPSSG NTAYA	588	CARDEGAG YYYYYMDV W	766	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-C01	Fzd2	FTFSNYAMT	216	SAIGTGGG TYYA	595	CATAYRRPG GLDVW	969	RSSQSLLHS DGKTYLY	1134	LGSNRAS	1237	CMQNTHW PLTR	1293
014S-F01	Fzd4	FTFSSYAMH	227	SVISTSGDT VLYT	652	CARGGSSD VR	838	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-H01	Fzd4	FTFSNYGM H	217	SYISSSSTI YYA	672	CARAALGYC TGGVCPPV DYW	737	RASQGISNN LN	1072	AASRLQS	1171	CQQSYSPP LTF	1363
014S-C03	Fzd7	YTFTNNFMH	383	GIIZPGGGR TIYA	517	CAKGDYGAL DYW	712	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-D03	Fzd7	FNFSSYTMR	173	SVIYGGGN TNYA	653	CARGGSGG NLSYW	836	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-F03	Fzd8	YTFTNNFMH	383	GGIIPLFGT ANYA	506	CARLVVRG GYGMDVW	893	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-A04	Fzd8	GTFSSYAIS	295	GWISSFNG NTKYA	575	CARADDYY DSSGYYYGF DYW	736	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-C04	Fzd8	FTFSSYTMN	235	SRINGDGS NTNYA	623	CARGWAGF DYW	871	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-D04	Fzd8	HTFSGYHIH	312	GWINAGNG NTTYA	558	CARDLSPMV RGVISGMDV W	779	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372
014S-E05	Fzd8	YTFTNNFMH	383	GIISPGGGR TIYA	515	CAKGDYGAL DYW	712	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP LTF	1372

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
014S-E06	Fzd10	FTFGNYDM N	195	SSLSWNSG TIVYA	641	CARDSSSG WYASYYGM DVW	798	RASQSISRY LN	1095	AASSLQS	1175	CLQHHSYP FTF	1275
027S-C02	Fzd5	YTLTTWYMX	398	GWMNPNS GNTAYA	582	CARGALGM DVW	819	QASQDISNY LN	1046	AASSLHT	1173	CQESYSSP YTF	1302
027S-E03	Fzd8	YTFTGHYM H	378	GWMNPNS GNTGYA	583	CARGTGGF DYW	867	RASQSISSYL N	1098	AASSLQS	1175	CQQSYSTP YTF	1379
027S-F03	Fzd8	YTFTGHYIH	377	GWMNPISG NTGYA	580	CARSTPFDP W	925	RASHDIGTFL A	1057	AASTLQS	1179	CQQSYRTP YTF	1355
027S-G03	Fzd8	YTFTHSYIH	380	GWINAKSG GTFYA	559	CARGDYDF WSGYHEYY YYGMDVW	824	QATQNIKKY LN	1051	KASTLES	1229	CQQSYSTP LTF	1372
027S-H03	Fzd8	YTFTSYYMH	392	GWINPNSG GTNYA	564	CARAPLDGS GSYYVDW	746	RSSQSLLHS NGYNYLD	1138	LGSNRAS	1237	CMQALQTP QTF	1262
027S-A04	Fzd8	YTFTNHFMH	382	GWISPNRG GTNYA	571	CARDCSGG SCYSHFDY W	765	RASQSISRS LA	1093	AASNLQS	1169	CQQAYSFP QTF	1323
027S-B04	Fzd8	FTVGSWYM S	259	SAIGTGGG TYYA	595	CAKDITPYG DYSILSHW	702	RASQAISNY LN	1059	AASSLQS	1175	CQQTFSPP LTF	1383
027S-C04	Fzd8	YTFTSHWM H	386	GGIIPIFGTT NYA	503	CARDSSSW YSYYYYYMD VW	799	RASQGINNY LA	1067	QASNLES	1243	CQQTYSSP LTF	1393
027S-D04	Fzd8	YTFTTYFMH	393	GWIYPNSG GTKYA	578	CTTDLRYDS SGPAAFDIW	1019	QASQDIDNY LN	1040	AASSLQS	1175	CQQSYSTP VTF	1377
027S-E04	Fzd8	FTFSDHYMS	205	SGISGSGG TTYYA	609	CATYGDFGY FDLW	978	RASQGIRND LG	1069	AASTLQS	1179	CQQAYSFP WTF	1324
027S-C05	Fzd8	GSFSTSVFG	279	GRIIPLFGTT NYA	532	CVKDRAWG FDYW	1027	RASQGIRND LA	1066	AASSLQR	1174	CQQSYSKP TF	1359
027S-D05	Fzd8	YTFTSYYMH	392	GWINPKSG GTNYA	560	CARGGFVF DYW	828	QASQDISNY LN	1046	ASSTLQT	1182	CQQSYSAP YTF	1356
027S-E05	Fzd8	GTFSSYAIS	295	GMINPSGG STTYA	523	CARQAGLH CSSTSCYLG NWFDPW	905	RASQGITKS LA	1077	AASNLQL	1168	CQQYNTFP ITF	1422

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
027S-F05	Fzd8	GTFNRYGIS	290	GGIIPRLGA TDYA	508	CAKGNWAF DIW	719	RASQSISTYL A	1100	GASTRAT	1217	CQQYGSS PTF	1411
027S-G05	Fzd8	GTFSSYAIS	295	GWISPYNG NTKYA	572	CARGVWTT PMGGGGN WFDPW	869	RASQSISSYL N	1098	YASSLQN	1261	CQQSYSTP FTF	1368
027S-H05	Fzd8	GTFGNYGIN	286	GWINPNSG GTNYA	564	CARETTDYY YGMDVW	814	RASQSIGTY LN	1088	DASNLET	1185	CQQANSFP LTF	1317
027S-A06	Fzd8	GTFSSYAIN	294	GVIDPSTG GTNYA	550	CARVLPGDS SGWYRGYY YYYGMDVW	941	RASQGISNN LN	1072	KASSLES	1226	CQQANSFP ITF	1316
027S-C06	Fzd8	GTFTSYPIS	306	GWINTYNG NTIYA	566	CARDLDSGF DLW	776	RASQGVGD YLA	1078	DASNLQS	1186	CQQHNAY PLTF	1330
027S-D06	Fzd8	GTFSSYAIS	295	GWISAYNG HTNYA	569	CARGGYSY GTVFDYW	842	RASQDISSW LA	1064	KASTLES	1229	CQQSYGA PLTF	1348
027S-E06	Fzd8	YTFTKDYMH	381	GGIIPIFGTA NYA	499	CARGLPPAA GGGGYFQH W	847	RASQNVND WLA	1082	SASNLQS	1252	CQQSYSTP FTF	1368
027S-F06	Fzd8	FTFSSYAMH	227	AVTWYDGS NKYYA	480	CAKDLVPYC SGGSCPPS GW	705	RASQSISSYL N	1098	GASNLQS	1207	CQQSYSTP LTF	1372
027S-G06	Fzd5	YTFTDYYMH	376	GWMSPNS GNAGFA	584	CARGKSGS FDYW	846	RASQSVNNT YVA	1111	GTSTRAT	1222	CQQYDTSP PTF	1404
027S-H06	Fzd5	YTFTGHYIH	377	GIINPSGGS TSYA	513	CARGFCSG GSCLWYGM DVW	825	RASQSISSYL N	1098	AASSLQS	1175	CQQSYTTP FTF	1382
027S-A07	Fzd5	GTFGSYAIT	287	GGIIPIFGTA NYA	499	CAKDNGWY FDLW	706	RASQSISTN VN	1099	AASSLQS	1175	CQQSYSTP YTF	1379
029S-B01	Fzd1	GTFSSYAIS	295	GRINPHNG NTNYA	1461	CARATRVSA AGTVHFQH W	1472	KSSQSVLHS SNNKNYLA	1498	STNTRSS	1257	CQQYYSTP FTF	1544
029S-D01	Fzd2	YTFTRYYIH	1452	GWMNPNS GNTGYA	583	CARVRFLEE MDVW	1494	RASQSLSSW LA	1512	DASTLQS	1520	CQQAISFP LTF	1315
029S-C02	Fzd2	GTFSSYGIS	1457	GIINPSGGS TSYA	513	CARGDIVAT MGMKKVDY YYYMDVW	1478	RASQDISNN LN	1500	GASHLQT	1522	CQQANSFP VTF	1316

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
029S-F02	Fzd2	YTFTRYYLH	1453	GWMNPNS GNTGYA	583	CARGIGYW	1481	RASQGISNY LA	1074	AASRLQT	1515	CLQYNTYP WTF	1528
029S-H02	Fzd2	GTFSTYAIS	298	GDIIPIFGSA NYA	1458	CARELGLG WFDPW	1476	RSSQSLLHS NGYNYLD	1138	LGSSRAS	1525	CMQALQTP LTF	1281
030S-A02	Fzd7	YTFTDYYMH	1449	GWMNPNS GSTGYA	1467	CARGDINYG NFDYW	1477	RASQSISSYL N	1098	KASTLHN	1523	CQQAISFP LTF	1315
030S-B02	Fzd3	YTFTDYYMH	1449	GWMNPNS GNTGYA	583	CARQGGSY SMGLDPW	1488	RASQSITTYL N	1511	KTSSLQS	1524	CQQGDSF PYTF	1531
029S-E03	Fzd3	YTFTGYYMH	379	GWINPNSG NTGYA	1463	CARSYYGVI DAFDIW	1492	RASQSISSYL N	1098	AASSLQT	1516	CQQSFRLP LTF	1532
029S-G03	Fzd3	YTFTNYYMH	1451	GWMNPNS GNTGYA	583	CAREDDFW SGGGMDVW	1474	RASQSIISYL N	1509	AASSLQS	1175	CQQSWRF PYTF	1535
030S-E03	Fzd3	FTFSDYYMS	1438	SAISGSGHS TYYA	1468	CAREGLRG WSIFDIW	1475	KSSQSVLYS SNNKNYLA	1037	WASTRES	1526	CQQYYSTP PTF	1545
029S-D05	Fzd3	YTFTDHYFH	1447	GWANPSSG NTGTA	1462	CARSRLRW DWYFDLW	1491	RASQTISSYL N	1513	DASNLET	1185	CQQSYSIP LTF	1536
030S-H03	Fzd3	FSFSSHAMS	1437	SAIGTGGG TYYA	595	CANPKHYW	1470	RASQGVSTY LA	1505	AASSLQS	1175	CQQYYSSP QTF	1543
029S-B06	Fzd3	YTFSRHYIH	1446	GWMNPNS GNTGYA	583	CARGGHTG YSSGWYNH W	1479	RASQSVSS WLA	1120	AASSLQS	1175	CQQAFRFP PTF	1529
029S-E06	Fzd3	YWFTASYM H	1455	GWMKPDS GNTGYA	1465	CARRSSSW GWYFDLW	1489	RASQNINSW LA	1506	AASSLQS	1175	CQQYYSFP LTF	1542
029S-H06	Fzd3	YTFAKYYIH	1444	GWMNPNS GNTGYA	583	CARHKRHTP YAFDIW	1487	RASQSISSYL N	1098	AASSLQS	1175	CQQSHSTP LTF	1533
029S-G07	Fzd3	YTFTDSYIH	1448	GWISAYNG NTNYA	570	CARGSGYF DLW	1486	RASQSISKW LA	1510	GASTLQS	1215	CQQAYSFP WTF	1324
029S-H08	Fzd3	YTFTGHYM H	378	GWMNPNS GNTGYA	583	CARVGDYD RFNWYFDL W	1493	RASRTVYNF LA	1514	DASNLRT	1519	CQQSYSTP PTF	1537
029S-F09	Fzd3	GTFSSYAIT	1456	GWISAYNG NTNYA	570	CARANRGL RKNYYYGM DVW	1471	RASQSIARY LN	1507	GASSLQS	1211	CQQSYNTP WTF	1351

Clone ID	Initial Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
030S-F04	Fzd3	YTFTSSYIH	1454	GIINPSGGGAVYA	1459	CARWTTVVTGAAFDIW	1495	RASQGIRNDLN	1502	DASNLGT	1518	CQQSSRIPPTF	1534
029S-A10	Fzd3	YSFTGYYLH	1442	GWINPNSGGTNYA	564	CARDHGTMIAVAGTFDYYYMDVW	1473	RASQGISKYLA	1503	AASSLQS	1175	CQQSYSTPWTF	1538
029S-B11	Fzd3	YTFNGYYMH	1445	GIVNPSGGGTNYA	1460	CARGGNYGRWLQPWYFDLW	1480	QASQDISNYLN	1046	GASALRS	1521	CQQTKSFPLTF	1540
029S-D11	Fzd3	HTFTSHYMH	1440	GWMNPNSANAGYA	1466	CARGLGYFDLW	1483	RASQDISRGLG	1501	AASTLYR	1517	CQQAYSFPWTF	1324
030S-H05	Fzd7	YSFTNYYMH	1443	GWMNPNSGNTGYA	583	CARSPDFWSGEGYFDLW	1490	RASQSIGNYLN	1508	AASSLQS	1175	CQQANSFPLTF	1530
030S-A06	Fzd7	YMFTGHDMH	1441	GRIIPILGIANYA	529	CARGIHGDYGLDYYYMDVW	1482	RASQAIGRRLA	1499	AASSLQS	1175	CQQYDTYWTF	1541
029S-C12	Fzd7	YTFTGYYMH	379	GWMNPNSGNTGYA	583	CARGMEYW	1484	RASQGISSYLA	1076	AASTLQS	1179	CLQYNTYPWTF	1528
030S-C06	Fzd7	YTFTGYYIH	1450	GWMDPNSGYTGYA	1464	CARGPADFWSGYKNDYFDFW	1485	RASQGISSWLA	1504	DASSLQS	1190	CQQSYSTPYSF	1539
4A12	Fzd5	GYTFTNYDIN	1439	WIYPRDGSTKYNEKFKG	1469	CVRSAWGFAY	1496	KASQDVGTAVA	1497	WASTRHT	1527	QQYSTYPLT	1546

Table 1B: Anti-Fzd Antibody Clone IDs, Heavy Chain (HC) and Light Chain (LC) Seq ID Nos, and Binding Characteristics

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
001S-B01	1	38	Fzd1,2,7,9
001S-E02	2	39	Fzd1,2,7
001S-G02	3	40	Fzd1,2,7
001S-H02	4	41	Fzd1,2,7
001S-A03	5	42	Fzd1,2,7,9
001S-B03	6	43	Fzd1,2,7
004S-G06	7	44	Fzd5,8
002S-B01	8		Fzd1
002S-C02	9		Fzd1
002S-E02	10		Fzd1
002S-G02	11		Fzd1
002S-F03	12		Fzd1
002S-A04	13		Fzd1
002S-B04	14		Fzd1
002S-D04	15		Fzd1
004S-H04	16	45	Fzd5
001S-A04	17	46	Fzd1,2,5,7,8
003S-E07	18	47	Fzd2
003S-D10	19	48	Fzd4
004S-B08	20	49	Fzd6
004S-D08	21	50	Fzd6
004S-C09	22	51	Fzd6
004S-F10	23	52	Fzd6
004S-A11	24	53	Fzd6
004S-A12	25	54	Fzd6
005S-B07	26	55	Fzd9

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
005S-D08	27	56	Fzd9
005S-E09	28	57	Fzd9
005S-H10	29	58	Fzd9
005S-B11	30	59	Fzd9
005S-D11	31	60	Fzd9
014S-G02	32	61	Fzd6
014S-B04	33	62	Fzd8
014S-B06	34	63	Fzd9
014S-G06	35	64	Fzd10
014S-A07	36	65	Fzd10
017S-B09	37		Fzd8
004S-D01	129	130	Fzd4
004S-E09	131	132	Fzd6
004S-F09			Fzd6

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
004S-H09			Fzd6
004S-B10			Fzd6
004S-C10			Fzd6
004S-F10			Fzd6
004S-G10			Fzd6
004S-A11			Fzd6
004S-B11			n.b.
004S-D11			Fzd6
004S-E11			n.b.
004S-F11			Fzd6
004S-G11			Fzd6
004S-A12			Fzd6
004S-B12			Fzd6

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
004S-C12			n.b.
004S-D12			n.b.
004S-F12			n.b.
004S-F12			n.b.
004S-G12			n.b.
005S-B02			n.b.
005S-C02			n.b.
005S-D02			Fzd5,8
005S-E02			Fzd5,8
005S-H02			Fzd5,8
005S-A03			Fzd5,8
005S-C03			n.s.
005S-E03			n.s.

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
005S-F03			Fzd8
005S-B04			Fzd5,8
005S-F04			n.b.
005S-G04			Fzd5,8
005S-H04			n.b.
005S-E05			n.b.
005S-G05			Fzd5,8
005S-H05			Fzd5,8
005S-D06			Fzd8
005S-F06			n.b.
005S-G06			n.b.
005S-A07			Fzd9,10
005S-B07			Fzd9

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
005S-A08			Fzd9
005S-B08			Fzd9
005S-D08			Fzd9
005S-E08			Fzd9
005S-F08			n.b.
005S-C09			Fzd9
005S-D09			Fzd9
005S-E09			Fzd9
005S-F09			Fzd9
005S-A10			Fzd9
005S-B10			Fzd9
005S-E10			Fzd9
005S-H10			Fzd9

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
005S-B11			Fzd9
005S-D11			Fzd9
005S-G11			n.b.
005S-H11			n.b.
005S-E12			Fzd10
006S-A01			Fzd10
006S-H01			n.b.
006S-A02			Fzd10
006S-D02			n.b.
006S-H02			Fzd10
006S-A03			n.b.
006S-B03			n.b.
006S-C03			n.b.

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
014S-A01			Fzd1,2,7
014S-B02			n.b.
014S-G02			Fzd6
014S-B03			n.b.
014S-C03			Fzd1,2,7
014S-A04			n.b.
014S-B04			Fzd8
014S-B05			Fzd5,8
014S-B06			Fzd9
014S-F06			n.s.
014S-G06			Fzd10
014S-A07			Fzd10
017S-E08			Fzd8

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
017S-H08			n.b.
017S-A09			n.b.
017S-B09			Fzd8
018S-F06			Fzd4
018S-H06			n.b.
018S-B07			n.b.
017S-A10			n.b.
017S-B10			n.b.
017S-D10			n.b.
018S-H08			n.b.
018S-B09			Fzd5,8
021S-A01			n.b.
021S-E02			Fzd5,8

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
021S-G02			n.s.
021S-A03			n.b.
029S-B01			n.b.
029S-D01			Fzd1,2,7
029S-C02			Fzd1,2,7
029S-H02			Fzd1
030S-A02			Fzd7
029S-E06			Fzd2, 6, 3
030S-F04			Fzd3
030S-H05			Fzd7
030S-A06			Fzd1,2,7,5
029S-C12			Fzd7
030S-C06			Fzd1

Clone ID	HC SID NO	LC SID NO	Confirmed Binding
001S-A01			Fzd1,2,7
001S-H01			Fzd1,2,7

In certain embodiment, the Fzd binding domain may be selected from any binding domain that binds a Fzd with an affinity of, e.g., a K_D of at least about 1 x 10^{-4} M, at least about 1 x 10^{-5} M, at least about 1 x 10^{-6} M, at least about 1 x 10^{-7} M, at least about 1 x 10^{-8} M, at least about 1 x 10^{-9} M, or at least about 1 x 10^{-10} M. In certain embodiment, the Fzd binding domain may be selected from any binding domain that binds one or more Fzd receptor at high affinity, e.g., a K_D of less than about 1 x 10^{-7} M, less than about 1 x 10^{-8} M, less than about 1 x 10^{-9} M, or less than about 1 x 10^{-10} M. In certain embodiment, the Fzd binding domain may be selected from any binding domain that binds Fzd at high affinity, e.g. a K_D of less than or equal to about 1 x 10^{-4} M, less than or equal to about 1 x 10^{-5} M, less than or equal to about 1 x 10^{-6} M, less than or equal to about 1 x 10^{-7} M, less than or equal to about 1 x 10^{-8} M, less than or equal to about 1 x 10^{-9} M, or at least about 1 x 10^{-10} M in the context of a Wnt surrogate molecule.

Suitable Fzd binding domains include, without limitation, de novo designed Fzd binding proteins, antibody derived binding proteins, e.g. scFv, Fab, etc. and other portions of antibodies that specifically bind to one or more Fzd proteins; VHH or single domain antibody derived binding domains; knottin-based engineered scaffolds; norrin and engineered binding fragments derived therefrom, naturally occurring Fzd binding domains, and the like. A Fzd binding domain may be affinity selected to enhance binding to a desired Fzd protein or plurality of Fzd proteins, e.g. to provide tissue selectivity.

In some embodiments, the Fzd binding domain binds to one, two, three, four, five or more different frizzled proteins, e.g. one or more of human frizzled proteins Fzd1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7, Fzd8, Fzd9, Fzd10. In

some embodiments, the Fzd binding domain binds to Fzd1, Fzd2, and Fzd 7. In some embodiments, the Fzd binding domain binds to Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8. In other embodiments the Fzd binding domain is selective for one or more frizzled protein of interest, e.g. having a specificity for the one or more desired frizzled protein of at least 10-fold, 25-fold, 50-fold, 100-fold, 200-fold or more relative to other frizzled proteins.

In certain embodiments, the Fzd binding domain comprises the six CDR regions of the pan specific frizzled antibody OMP-18R5 (vantictumab). In certain embodiments, the Fzd binding domain is an scFv comprising the six CDR regions of the pan-specific frizzled antibody OMP-18R5 (vantictumab). See, for example, U.S. Patent no. 8507442, herein specifically incorporated by reference. For example, the CDR sequences of OMP-18R5 include a heavy chain CDR1 comprising GFTFSHYTLS (SEQ ID NO:270), a heavy chain CDR2 comprising VISGDGSYTYYADSVKG (SEQ ID NO:677), and a heavy chain CDR3 comprising NFIKYVFAN (SEQ ID NO:1033), and (ii) a light chain CDR1 comprising SGDKLGKKYAS (SEQ ID NO:1152) or SGDNIGSFYVH (SEQ ID NO:1153), a light chain CDR2 comprising EKDNRPSG (SEQ ID NO:1200) or DKSNRPSG (SEQ ID NO:1201), and a light chain CDR3 comprising SSFAGNSLE (SEQ ID NO:1435) or QSYANTLSL (SEQ ID NO:1436). In particular embodiments, the Fzd binding domain is an antibody or derivative thereof, including without limitation scFv, minibodies, VHH or single domain antibodies (sdAb) and various antibody mimetics comprising any of these CDR sequences. In certain embodiments, these CDR sequences comprise one or more amino acid modifications.

In other embodiments, the Fzd binding domain comprises a variable region sequence, or the CDRs thereof, from any of a number of frizzled specific antibodies, which are known in the art and are commercially available, or can be generated de novo. Any of the frizzled polypeptides can be used as an immunogen or in screening assays to develop an antibody. Non-limiting examples of frizzled binding domains include antibodies available from Biolegend, e.g. Clone

CH3A4A7 specific for human frizzled 4 (CD344); Clone W3C4E11 specific for human Fzd9 (CD349); antibodies available from Abcam, e.g. ab64636 specific for Fzd7; ab83042 specific for human Fzd4; ab77379 specific for human Fzd7; ab75235 specific for human Fzd8; ab102956 specific for human Fzd9; and the like. Other examples of suitable antibodies are described in, inter alia, US Patent application 20140105917; US Patent application 20130230521; US Patent application 20080267955; US Patent application 20080038272; US Patent application 20030044409; etc., each herein specifically incorporated by reference.

The Fzd binding region of a Wnt surrogate molecule may be an engineered protein that is selected for structural homology to the frizzled binding region of a wnt protein. Such proteins can be identified by screening a structure database for homologies. The initial protein thus identified, for example the microbial Bh1478 protein. The native protein is then engineered to provide amino acid substitutions that increase affinity, and may further be selected by affinity maturation for increased affinity and selectivity in binding to the desired frizzled protein. Non-limiting examples of frizzled binding moieties include the Fz27 and Fz27-B12 proteins.

In particular embodiments, a Wnt surrogate molecule comprises an LRP5/6 binding domain, e.g., an anti-LRP5/6 antibody, or antigen-binding fragment thereof, fused to a polypeptide that specifically binds to one or more Fzd receptors. In particular embodiments, the polypeptide that specifically binds to LRP5/6 is an antibody or antigen-binding fragment thereof. If certain embodiments, it is an antibody or antigen-binding fragment thereof disclosed in the U.S. provisional patent application no. 62/607,879, titled, "Anti-LR5/6 Antibodies and Methods of Use," Attorney docket number SRZN-005/00US, filed on December 19, 2017, which is incorporated herein by reference in its entirety. In particular embodiments, the LRP5/6 binding domain comprises the three heavy chain CDRs and/or the three light chain CDRs disclosed for any of the illustrative antibodies or fragments thereof that bind to LRP5 and/or LRP6 provided in Table 2A. In particular

embodiments, the LRP5/6 binding domain comprises the three heavy chain CDRs and/or the three light chain CDRs disclosed for any of the illustrative antibodies or fragments thereof that bind to one or more Fzd receptor provided in Table 2A, wherein the CDRs collectively comprise one, two, three, four, five, six, seven, or eight amino acid modifications, e.g., substitutions, deletions, or additions. In certain embodiments, the LRP5/6 binding domain is a VHH or sdAb or was derived from a VHH or sdAb, so Table 2A only includes the three heavy chain CDRs. In certain embodiments, the LRP5/6 biding domain comprises the three heavy chain CDRs shown in Table 2A or variants wherein the CDRs collectively comprise one, two, three, four, five, six, seven or eight amino acid modifications. In particular embodiments, the LRP5/6 binding domain comprises the heavy chain fragment and/or light chain fragment of any of the illustrative antibodies or fragments thereof that bind to LRP5 and/or LRP6 provided in Table 2B or SEQ ID NOs:66-88 or 133 (or an antigen-binding fragment or variant of either). In certain embodiments, the LRP5/6 binding domain is an Fab or was derived from an Fab, so Table 2B includes VH and CH1 sequence, but not CH2 or CH3 sequences. In certain embodiments, the LRP5/6 binding domain is a VHH or sdAb or was derived from a VHH or sdAb, so Table 2B includes the VHH domain. In certain embodiments, the LRP5/6 binding region is a polypeptide, e.g., an antibody or antigen-binding fragment thereof, that competes with one of these antibodies for binding to LRP5 and/or LRP6.

Table 2A: Anti-LRP5/6 Antibody Clone IDs and CDR sequences.

Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
001S-C08	LRP6e1e2	YTISNYYIH	1682	GMINPSGGSTTYA	1762	CAIVRGKKWYFDLW	1842	RASQYISNYLN	2098	AASSLQS	2110	CQQSYITPLTF	2160
001S-C10	LRP6e1e2	RTFGTYPNG	1632	AAISWGGRTAYA	1700	CYARTVIGGFGAFRAHW	2061						
001S-D10	LRP6e1e2	RTFSRYAMA	1642	AAIRWSGGGTYYA	1689	CAASMEAMNSLRVNKERYYQSW	1836						
001S-E10	LRP6e1e2	LTFSNAAMA	1614	AAISRSGANTAYS	1696	CTLVNEIKTWW	2039						
001S-F10	LRP6e1e2	RTFSSYAMA	1645	AAIKWSGTNTYYA	1684	CAASMEAMNSLRVNKERYYQSW	1834						
001S-G10	LRP6e1e2	RTFSRYVMG	1644	AAITWRGGSTYYA	1706	CATGPNSIY	1987						
001S-A11	LRP6e1e2	RTFGNYDMG	1630	AGIRWSGSTLYA	1709	CYARTVIGGFGAFRAHW	2062						
001S-B11	LRP6e1e2	RRFTTYGMG	1623	AAVTWRSGSTYYA	1708	CAAGSTVVAEFNYW	1828						
001S-C11	LRP6e1e2	SISSFNTMG	1659	AVITTGGDTSYS	1741	CNKVNAITKL	2025						
001S-E11	LRP6e1e2	RTLSRYSMG	1651	AAISRSGDRIYYS	1697	CTLVNEIKTWW	2040						
001S-F11	LRP6e1e2	RTFSSYAMS	1646	AVIGRSGGIKYYA	1736	CATRRPFNSYNTEQSYDSW	1989						
001S-G11	LRP6e1e2	SIFRLGTMY	1655	ASIGKSGSTNYA	1719	CKQHPNGYR	2005						
001S-H11	LRP6e1e2	RTLSSFAMG	1652	ATISRSGGNTYYA	1732	CNLREWNNSGAGYW	2026						
001S-A12	LRP6e1e2	IAFRYYDMG	1608	AAITWNGRSSDYA	1704	CAAVFTGRFYGRPPREKYDYW	1838						
001S-B12	LRP6e1e2	RLLSYYALA	1622	AAISRNGDKSHYS	1694	CTLVNEIKTWW	2038						
001S-C12	LRP6e1e2	RTFSNYAVG	1641	AAISRFGGSTYYV	1693	CAADRIENYLGRYYDPSEYEYW	1824						

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
001S-D12	LRP6e1e2	RTFSRYAMG	1643	GAISRSGNNTYYA	1744	CTLVNEIKTWW	2041						
001S-F12	LRP6e1e2	RTFRSYTMG	1637	AAISGSGGSTTYA	1690	CNADIKTTTYSPLRNYW	2009						
008S-B01	LRP5	TIFSINTMG	1664	ATMTSGGNTNYA	1734	CYRRQWASSWGARNYEYW	2064						
008S-C01	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CHGRDYGSNAPQYW	2001						
008S-D01	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CYVKLRDDDYVYR	2065						
008S-E01	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CNAVTYNGYTIR	2023						
008S-G01	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CYARTQRMGVVNSYW	2060						
008S-A02	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CNAVTFGGNTIR	2021						
008S-C02	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CNAVTYDGY	2022						
008S-D02	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CAAQFRNDYGLRYQSTNNYW	1832						
008S-E02	LRP5	NINSIETLG	1617	ANMRGGGYMKYA	1716	CNANYRGNRYW	2019						
009S-C01	LRP6e3e4	GSFSGYYWT	1595	GEINHSGATNYN	1745	CVRYAWPEFDHW	2053	RASQRVSNYLN	2089	AASSLQG	2110	CQQSYSVPYTF	2178
009S-B02	LRP6e3e4	GSLSGYYWS	1596	GEINHSGSTNYN	1746	CVRYAWPEFDHW	2055	RASQSISNYLN	2090	AASSLQS	2110	CQQSYSLPLTF	2170
009S-C02	LRP6e3e4	GSFSDYYWS	1594	GEINHSGSTNYN	1746	CVRYAWPEFDHW	2054	RASQSISNYLN	2090	AASSLQS	2110	CQQSYSMPLTF	2171
009S-D02	LRP6e3e4	GTFSSYAIS	1603	GGIIPIFGTANYA	1749	CVYGRDFDYW	2056	SGSSSNVGNNYVS	2105	DNDKRPS	2122	CESWDSSLSSEVF	2139
010S-A02	LRP6e1e2	HTFSSYAMG	1607	AAISQSGYVRYYA	1691	CKIYGLNGQPLGSW	2003						
010S-B02	LRP6e1e2	RTFNSGTMG	1634	AAITWRGGITYYA	1705	CNADGYSWDGRSGRRLELW	2008						

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
010S-D02	LRP6e1e2	RTFSSYAVG	1647	AAISYSGGSTKYA	1702	CAASVYISRRDSDYGYW	1837						
010S-E02	LRP6e1e2	LSSGRPFSSYVMG	1612	AAISWSGGSTKYA	1701	CKLQVRPIGYSSAYSRNYW	2004						
010S-F02	LRP6e1e2	RSFNSYVIG	1625	AAIRWSGDNTYYA	1688	CAASMEAMNSLRVNKERYYQSW	1835						
009S-E02	LRP6e1e2	RRFTTYGMG	1623	AAVTWRSGSTYYA	1708	CAAGSTVVAEFNYW	1829						
009S-F02	LRP6e1e2	RTFSYYAMG	1649	AAISRSGGIYYA	1698	CNTVRPLWAW	2029						
009S-G02	LRP6e1e2	SIFSIYAMG	1658	AVITSGGKTVYA	1740	CYADSRSSWYDEYLEHW	2058						
009S-H02	LRP6e1e2	SIVRSLPMA	1660	ATINDAQRYYA	1727	CNTSPYMHDVW	2027						
009S-A03	LRP6e1e2	RTFSVYGVG	1648	AAVSASGGYTWYA	1707	CKAAPRWGGATAYW	2002						
010S-G02	LRP6e1e2	SIVRSLPMA	1660	ATINDAQRYYA	1727	CNTSPYMHDVW	2028						
010S-A03	LRP6e1e2	RTFRRYAMG	1636	ATISASGGNTAYA	1731	CNAPAWLYDDDYW	2020						
009S-B03	LRP6e1e2	RTFSNYAVG	1641	AAISRFGGSTYYV	1693	CAADRIENYLGRYYDPSEYEYW	1825						
010S-B03	LRP6e1e2	RTFSNYAVG	1641	AAISRFGGSTYYA	1692	CHAKQLRNGQMYTYW	1999						
010S-D03	LRP6e1e2	ISSVYGMG	1609	AAIQWSADNTFYA	1686	CAARTSGGLFHYRRSDHWDTW	1833						
009S-C03	LRP6e1e2	LPFSRYAMA	1610	AGMSGEGRNTKYR	1713	CSSRGYW	2034						
009S-D03	LRP6e1e2	SIFSDGAMG	1656	AVISGGRTGYA	1737	CNTYPFPIYKKGYPFW	2030						
009S-E03	LRP6e1e2	RRFTTYGMG	1623	AAVTWRSGSTYYA	1708	CAAGSTVVAEFNYW	1830						
009S-F03	LRP6e1e2	RTFSSYAMS	1646	AVIGRSGGIKYYA	1736	CATRRPFNSYNTEQSYDSW	1990						

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
010S-E03	LRP6e1e2	RSVSIYPMG	1628	AAINWSGDSTKYA	1685	CNAVVVGLSRRIDNIW	2024						
010S-F03	LRP6e1e2	RTFSRYVMG	1644	AAITWRGGSTYYA	1706	CATGPNSIY	1988						
009S-G03	LRP6e1e2	RSVSSYNMG	1629	AAISRRGGIIEYG	1695	CHAVENILGRFVDYW	2000						
009S-H03	LRP6e1e2	SIFSINTMG	1657	AVITSGGKTVYA	1740	CYADSRSSWYDEYLEHW	2057						
009S-A04	LRP6e1e2	RTLSAYDMG	1650	GGIRWSGGTTLYP	1753	CYARTVIGGFGAFRAHW	2063						
009S-B04	LRP6e3e4	SIFMINTMA	1654	ATIRPVVSETTYA	1728	CNAKRPWGTRDEYW	2018						
010S-G03	LRP6e3e4	RSFNSYTTT	1624	AAIRGSSGSTFYA	1687	CNAASTVTAWPYYGPDYW	2006						
009S-C04	LRP6e3e4	FRFSISTMG	1553	AYITGGGRTMDG	1743	CNAFVRSDFDRYYDYW	2011						
009S-D04	LRP6e3e4	TIVSIYRIN	1665	AGITSSGRTIYA	1712	CNAASTVTAWPYYGPDYW	2007						
010S-H03	LRP6e3e4	RIFSIYDMG	1621	SGIRWSGGTSYA	1789	CSSRGYW	2035						
009S-E04	LRP6e3e4	RIFAIYDIA	1618	AMIRPVVTEIDYA	1715	CNAKRPWGSRDEYW	2012						
010S-A04	LRP6e3e4	SLFSFNAVG	1662	ASISSGGRTNYA	1722	CSKGGVYGGTYVPDSW	2032						
009S-F04	LRP6e3e4	RSLSSFAMG	1627	ARISRGDGYTDEA	1718	CAAVQAVIGGTLTTAYDYW	1839						
010S-B04	LRP6e3e4	RVLSYYAMA	1653	AGITRGGATTYYS	1711	CAAGPNWSTRNREYDYW	1827						
009S-G04	LRP6e3e4	GTFSRYHMG	1601	SAITWSGGRTYYA	1788	CALTWAPTPTNRRSDYAYW	1872						
009S-H04	LRP6e3e4	RIFAIYDMA	1619	ATIRPVVSETTYA	1728	CNAKRPWGTRDEYW	2017						
010S-C04	LRP6e3e4	SLFSFNAMG	1661	ASISSGSRTNYA	1723	CSKGGVYGGTYVPDSW	2033						

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
010S-D04	LRP6e3e4	RIFAIYDIA	1618	ATIRPVVTQIDYA	1730	CNAKRPWGSRDEYW	2015						
010S-E04	LRP6e3e4	RTFGSDVMG	1631	ALTGWGDGSTTYYE	1714	CAAARRSGTYDIGQYLRESAYVFW	1820						
010S-F04	LRP6e3e4	RTFSRYAMG	1643	AAITRSGSNTYYA	1703	CAADPRGVTLPRATAYEYW	1823						
009S-A05	LRP6e3e4	RTFSDYSMG	1639	AGISWIADNRYYA	1710	CTAGRSRYLYGSSLNGPYDYW	2036						
010S-G04	LRP6e3e4	VIFALYDIA	1666	ATIRPVVTETDYA	1729	CNAKRPWGSRDEYW	2014						
010S-H04	LRP6e3e4	RSFSDFFMG	1626	ATISWSGSSANYE	1733	CAAAYSYSQYGSSYSYW	1821						
010S-A05	LRP6e3e4	LSFSSYAMG	1611	AAISRSGVSTYYA	1699	CAAKFGVLATTESRHDYW	1831						
010S-C05	LRP6e3e4	RTFNIDDMG	1633	ASIRWSGQSPYYA	1720	CNAETYSGNTIW	2010						
010S-D05	LRP6e3e4	RTFSDYSMA	1638	AGISWIADNRYYA	1710	CAGDRSRYLYGDSLRGPYGYW	1841						
010S-E05	LRP6e3e4	SVFTTFAKG	1663	ASITASSDRTFYA	1725	CAAYSTFNTDVASMKPDYW	1840						
010S-F05	LRP6e3e4	RIFSIYDIA	1620	ATIRPVVTETDYA	1729	CNAKRPWGSRDEYW	2013						
013S-G04	LRP6e3e4	RIFAIYDIA	1618	ATIRPVVSETTYA	1728	CNAKRPWGTRDEYW	2016						
013S-H04	LRP6e3e4	RTFSMYDMG	1640	ASIRWSSGNTWYA	1721	CYANIYYTRRAPEEYW	2059						
013S-A05	LRP6e3e4	RTFNTYAMG	1635	ASVSWRYDRTYYT	1726	CAADTNWRAGPRVGIDEYAYW	1826						
013S-B05	LRP6e3e4	FAFSTTAMS	1549	STINPGGLSKSYA	1806	CTKGGIQ	2037						
013S-C05	LRP6e3e4	NIFPIDDMS	1616	ATVTSGGRINYA	1735	CNVDRTLYGKYKEYW	2031						
013S-D05	LRP6e3e4	RIFSIYDMG	1621	SGIRWSGGTSYA	1789	CGSRGYW	1998						

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
013S-E05	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTADYA	1748	CARDWELYGMDVW	1907	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
013S-F05	LRP6e3e4	GTFSSYAIS	1603	GIINPSGGSTSYA	1761	CARAGYYDSSGYYAFDIW	1882	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
013S-G05	LRP6e3e4	YTFTYRYLH	1681	GGVIPIFGTADYA	1755	CASDIVVDDAFDTW	1969	RASQDISNYLN	2079	AASTLQS	2113	CQQGNSFPYTF	2152
010S-G06	LRP6e3e4	FSFETYGMS	1555	SGISGSGGRTHYA	1792	CARDLDYW	1897	QASQDISNYLN	2077	AASSLQS	2110	CQQSYRIHWTF	2163
009S-B05	LRP6e3e4	FTFDAYAMH	1560	STLSGDANNAYYA	1811	CARGGSGWSNYYGMDVW	1931	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-C05	LRP6e3e4	YTFTYRYLH	1681	GRIIPVLKITNYA	1768	CAVVDDAFDIW	1996						
009S-D05	LRP6e3e4	FTLRNHWLS	1591	SAISGSGGSTYYA	1786	CATRTGYSYGFNFWAFDIW	1991	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-E05	LRP6e3e4	YTFTNNFMH	1676	GHVDPGDGETIYA	1756	CARDWGIAAAGDYYYYGMDVW	1908	RASQGINSYLA	2081	DAKGLHP	2114	CQQSYSAPLSF	2166
009S-F05	LRP6e3e4	FTFDDYGMS	1561	SAIGTGGGTYYA	1781	CARLGSYGSPYYYYGMDVW	1959	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-G05	LRP6e3e4	FTFSDYYMS	1568	SGVSWNGSRTHYA	1799	CAKDSGLV	1852	QASQDISNYLN	2077	AASTLQR	2112	CQQSYSAPLTF	2167
009S-C06	LRP6e3e4	YTFASYDIH	1671	GWMNPNSGNTGYA	1776	CARATGSGWYTDLGYW	1883	RASRNINRYLN	2099	AASSLLS	2109	CQQSYNVPFTF	2162
009S-D06	LRP6e3e4	FTFSSHSTH	1573	STISDTNSGTYYA	1807	CAKAQATGWSGYYTFDYW	1843	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-E06	LRP6e3e4	FTFTDYGLH	1587	AVISYGGSNKYYA	1739	CASGYSYGLYYYGMDVW	1973	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-F06	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CATEAALDAFDIW	1985	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-G06	LRP6e3e4	YIFTDYYMH	1669	GWINPNSGGTNYA	1774	CARDFLGSTGDYW	1892	RASQNIGLYLN	2088	DASSLQR	2121	CQQSYSTPYTF	2176
009S-H06	LRP6e3e4	FTFSSSAMH	1574	SAIGTGGSTYYA	1783	CAKGGDYFYYYYGMDVW	1856	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-A07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CATAYGSSSLNIDYW	1980	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
009S-B07	LRP6e3e4	YTFTGYYMH	1675	GWINPNSGGTNYA	1774	CVKDGGSFPLAYAFDIW	2049	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-D07	LRP6e3e4	FPFRYYGMS	1551	ARIGWNGGSIVYA	1717	CARDYSDRSGIDYW	1910	RSSQSLLHSNGYNYLD	2102	LGSNRAS	2131	CMQATQFPLTF	2146
009S-F07	LRP6e3e4	GTFSSYAIS	1603	GIINPSGGSTSYA	1761	CARAAGNFWSGYYTFDYW	1876	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-G07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CARGSYGMDVW	1947	RASQGISNYLA	2083	DASNLET	2115	CLQDFSFPWTF	2140
009S-H07	LRP6e3e4	YTFTGYYMH	1675	GWMNPNSGNTGYA	1776	CASSVVPAGPAGVYAFDIW	1975	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-A08	LRP6e3e4	GTFSSHAIN	1602	GWISANNGNTDYA	1775	CARDQDYGDYGWYYYGMDVW	1902	RASQGISNYLA	2083	GSSTLQS	2127	CQQTYSIPPTF	2181
011S-C01	LRP6e3e4	LTFTSHGMS	1615	SYVSDSGSSVYYA	1818	CARHPGSFGGYSYAWYYYYGMDVW	1956	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-C08	LRP6e3e4	FSFNTFGIH	1556	AVISYDGSNKYYA	1738	CAKSIAAAGTGYYGMDVW	1868	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-D08	LRP6e3e4	YTFTSYDIN	1679	GGIIPIFGTANYA	1749	CARGPYYFDYW	1939	RASQGISNNLN	2082	DASSLES	2120	CLQHNSYPFTF	2143
011S-F01	LRP6e3e4	FSFSDYYMS	1558	SGISESGGRTYYA	1790	CASAADFDYW	1966	RASQDISNYLN	2079	AASSLQS	2110	CLQDYSYPRTF	2141
009S-E08	LRP6e3e4	YGFTGYYIH	1668	GWMNPNSGNTGYA	1776	CARGYGDYDLW	1951	QASQDISNYLN	2077	DASSLES	2120	CQQSYRYPTF	2165
009S-F08	LRP6e3e4	DTFANYGFS	1547	GXVNAGNGNTTYA	1777	CAKGWLDFDYW	1866	QASQDISNYLN	2077	DASSLES	2120	CQQSYSTSITF	2177
009S-G08	LRP6e3e4	FTFSDFAMT	1566	SYISGDSGYTNYA	1813	CARLGSYPGPYYYYMDVW	1961	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-H08	LRP6e3e4	YTFTDYFMN	1673	GIINPSGDSTRFA	1758	CARDDGLGGMDVW	1888	QASQDISNYLA	2076	AASSLQS	2110	CQQSYSTPLTF	2174
009S-A09	LRP6e3e4	YTFTYRYLH	1681	GRIIPILGSTNYA	1767	CTTDLWDYW	2047	QASQGITNYLN	2078	AASSLQS	2110	CLQDYTDPFTF	2142
011S-F02	LRP6e3e4	FTFSTYGMH	1584	SSISVSSGTTHYA	1804	CARGGSGSYYYAFDIW	1929	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-G02	LRP6e3e4	YTFTSYAMN	1678	GGIIPIFGTANYA	1749	CARDASGGSTGWYYFDSW	1886	RASQGISSYLA	2087	AASSLQS	2110	CQQAYSFPWTF	2150

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
011S-A03	LRP6e3e4	FTFSSYWMH	1580	STISGSGGRTYYA	1808	CATSPYGVFTLDYW	1993	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-C03	LRP6e3e4	YTFSYRYLH	1672	GGIIPIFGTANYA	1749	CASTVTTDAFDIW	1977	QASQDISNYLN	2077	DASSLES	2120	CQQSYSFPPFTF	2168
011S-D03	LRP6e3e4	FSFDDYGMS	1554	SVISSGGTIYYA	1812	CARHLSSGYLSYYGMDVW	1954	RASQSISSYLA	2093	AASTLQS	2113	CQQSYSTPLTF	2174
011S-F03	LRP6e3e4	FTFSSYAMS	1577	SAISGSGGSTYYA	1786	CAKGGRDGYKGYFDYW	1859	KSSQSVLYTTTNRNHIA	2073	WASSRKS	2135	CQQYYSTPYTF	2189
011S-C04	LRP6e1e2	GTFNSNAIS	1598	GWMNPNSGNTGYA	1776	CARDYYGSGSYNYGMDVW	1912	GASQSVPRNSLA	2066	GASQRAT	2124	CQQYHNWPPEYTF	2184
011S-D04	LRP6e1e2	YTFTSYDIN	1679	GIINPSGGSTSYA	1761	CAREAYYYYYGMDVW	1915	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-H04	LRP6e1e2	YIFTDYYMH	1669	GRIIPILGRANYA	1765	CARGGYSTLDYW	1932	QASQDISNYLN	2077	AASTLQS	2113	CQQSFSTPRTF	2156
008S-F02	LRP5	YTFTNYCMH	1677	GIINPSDGSTSHA	1757	CAKDMVHLIVALAIDYW	1851	RSSQSLLHSDGYTYLY	2101	TLSYRAS	2134	CMQALEALFTF	2144
010S-C06	LRP6e1e2	FTFNSYSMD	1563	SSISPRGGSTYYA	1802	CAPYYYDKSAKPLRSYFDHW	1875	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-E06	LRP6e3e4	LTVSSNYMS	1615	SGISWNSGSIGYA	1796	CARGSDCSGGSCYYSFDYW	1944	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-F06	LRP6e3e4	FTFSSSWMH	1575	SAIGTGGGTYYA	1781	CAREVAVKDYYYYYMDVW	1921	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-H06	LRP6e3e4	YTFTSYDIN	1679	GRIIPILGRTNYA	1766	CAREERGATGRAFDIW	1918	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-A07	LRP6e3e4	FTFSSYAMH	1576	ASISSTSGSKYYA	1724	CAKTYYDFWSGYYTFDYW	1870	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-B07	LRP6e3e4	FTFSDYYMS	1568	SMISYNGGRAFYA	1800	CARGNPYYFDYW	1937	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-C07	LRP6e3e4	FTFSKTDMH	1569	STITTDSRGTYYA	1810	CAKGGDYYYYYYGMDVW	1858	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-D07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CANGLEDAYAFDIW	1873	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-D05	LRP6e3e4	FTLRNHWLS	1591	SAISGSGGSTYYA	1786	CATRTGYSYGFNFWAFDIW	1992	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
009S-E05	LRP6e3e4	YTFTNNFMH	1676	GHVDPGDGETIYA	1756	CARDWGIAAAGDYYYYGMDVW	1909	RASQGINSYLA	2081	DAKGLHP	2114	CQQSYSAPLSF	2166
010S-E07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CAKDDFSLYGMDVW	1845	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-F05	LRP6e3e4	FTFDDYGMS	1561	SAIGTGGGTYYA	1781	CARLGSYGSPYYYYGMDVW	1960	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-F07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CARLDYGETEGNGDW	1958	RASQSVYSNLA	2097	DTSNRAT	2123	CQQYNNWPPITF	2185
010S-G07	LRP6e3e4	FTFSSYAMH	1576	STISGSGGSTYYA	1809	CARAGYGRYYYGMDVW	1880	RVSQGISSYLN	2103	AASSLQS	2110	CQQTYTIPFTF	2182
009S-G05	LRP6e3e4	FTFSDYYMS	1568	SGVSWNGSRTHYA	1799	CAKDSGLV	1853	QASQDISNYLN	2077	AASTLQR	2112	CQQSYSAPLTF	2167
010S-H07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CARDDSMGAFDIW	1890	QASQDISNYLN	2077	GTSNLQS	2128	CQQSYSTPYTF	2176
010S-A08	LRP6e3e4	HTFLTYDIN	1606	GRITPRLGIANYA	1770	CASYFGVMDVW	1979	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-A07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CATAYGSSSLNIDYW	1981	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-B07	LRP6e3e4	YTFTGYYMH	1675	GWINPNSGGTNYA	1774	CVKDGGSFPLAYAFDIW	2050	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-B06	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CAPALTDAGSFDYW	1874	RVSQSISSYLN	2103	AASSLQS	2110	CQQSYSTPLTF	2174
010S-B08	LRP6e3e4	YTFTYRYLH	1681	GGIIPVFGTADYA	1751	CARDREQQILDYW	1904	RASQGISNNLN	2082	DASNLET	2115	CQQSYTSRLTF	2179
010S-C08	LRP6e3e4	FTFSTFGMH	1582	STITSSGGSTYYA	1809	CARAGIAAAPGSRNYYGMDVW	1878	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-C06	LRP6e3e4	YTFASYDIH	1671	GWMNPNSGNTGYA	1776	CARATGSGWYTDLGYW	1884	RASRNINRYLN	2099	AASSLLS	2109	CQQSYNVPFTF	2162
009S-D06	LRP6e3e4	FTFSSHSTH	1573	STISDTNSGTYYA	1807	CAKAQATGWSGYYTFDYW	1844	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-D08	LRP6e3e4	FTFSSSWMH	1575	SAIGTGGGTYYA	1781	CAKEDYDSSGYYYYYFQHW	1855	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-E06	LRP6e3e4	FTFTDYGLH	1587	AVISYGGSNKYYA	1739	CASGYSYGLYYYGMDVW	1974	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
010S-E08	LRP6e3e4	YSFTRTDMH	1670	GYISAYTGHTSYA	1778	CARDLGGTADYW	1898	RASQSISSYLN	2094	ZASSLQS	2137	CQQSYSTPLTF	2174
010S-F08	LRP6e3e4	LTFDDHAMH	1613	SYISSSGRTIFYA	1815	CVRGDSGWGILYYVMDVW	2052	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-F06	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CATEAALDAFDIW	1986	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-G08	LRP6e3e4	YIFTDYYMH	1669	GGFDPEDGETIYA	1747	CARGGGPNEHDYYFDYW	1927	RASQSVRSSDLA	2096	GSSSRAT	2126	CQQYGRSPRYSF	2183
010S-H08	LRP6e3e4	FTFZNAWMS	1590	SGISGSGGSTYYA	1793	CARGRGKKNYYYGMDVW	1942	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-A09	LRP6e3e4	FTFSTYYMS	1586	SGISWNGGKTHYV	1794	CARGGDFDYW	1925	QASQDIANYLN	2075	AASSLQS	2110	CQQSYSTPYTF	2176
010S-B09	LRP6e3e4	GTFSSYAIS	1603	GWINPNSGDTNYA	1773	CARGEQWLVWGFDPW	1924	RASQSISRYLN	2092	KASSLES	2130	CQQSYDSPWTF	2159
009S-G06	LRP6e3e4	YIFTDYYMH	1669	GWINPNSGGTNYA	1774	CARDFLGSTGDYW	1893	RASQNIGLYLN	2088	DASSLQR	2121	CQQSYSTPYTF	2176
010S-C09	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CARDEVEGGMDVW	1891	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-H06	LRP6e3e4	FTFSSSAMH	1574	SAIGTGGSTYYA	1783	CAKGGDYFYYYYGMDVW	1857	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-D09	LRP6e3e4	GTFSSYTIS	1603	GGIVPAYRRANYA	1754	CAKGGYELDYW	1865	QASQDISNYLN	2077	AASSLQS	2110	CQQIHSYPLTF	2155
010S-E09	LRP6e3e4	GDLSIYTIN	1593	GWINAGNGNTTYA	1772	CARGGDSSGYYYYAFDIW	1926	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-A07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CATAYGSSSLNIDYW	1982	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-B07	LRP6e3e4	YTFTGYYMH	1675	GWINPNSGGTNYA	1774	CVKDGGSFPLAYAFDIW	2051	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-D08	LRP6e3e4	YTFTSYDIN	1679	GGIIPIFGTANYA	1749	CARGPYYFDYW	1940	RASQGISNNLN	2082	DASSLES	2120	CLQHNSYPFTF	2143
010S-F09	LRP6e3e4	FTFDEYAMH	1562	STISGSGGSTYYA	1809	CASAKNDFWSGYFAFDYW	1968	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-G09	LRP6e3e4	GTFNTHTIT	1599	GWMNPNSGNTGYA	1776	CARGNLDFDYW	1936	QASQDISNYLN	2077	DASNLET	2115	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
010S-H09	LRP6e3e4	FTFSDHYMS	1567	SAISSGSDRTYYA	1787	CARYSGYDFDYW	1965	RASQGISNYLN	2084	AASTLQS	2113	CQQGYGTPPMF	2153
010S-A10	LRP6e3e4	FSFSSYSMN	1559	SYISSSSSTIYYA	1816	CARGSGYYGPGYYGMDVW	1946	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-D07	LRP6e3e4	FPFRYYGMS	1551	ARIGWNGGSIVYA	1717	CARDYSDRSGIDYW	1911	RSSQSLLHSNGYNYLD	2102	LGSNRAS	2131	CMQATQFPLTF	2146
010S-B10	LRP6e3e4	FAFKDYYMT	1548	SAIGAGGGTYYA	1779	CARESALYSSSWYYYYYGMDVW	1920	RASQSISSYLN	2094	GTSSLHT	2129	CQQANSFPFTF	2148
010S-C10	LRP6e3e4	FTFSSYAMS	1577	SAISGSGGSTYYA	1786	CAKGGRDGYKGYFDYW	1860	KSSQSILSSSSNRDSLA	2072	WASSRKS	2135	CQQYYNIPYSF	2187
009S-E07	LRP6e3e4	YTFTGYYIH	1674	ZHVDPEDGETIYA	1819	CARGPAAIGILGWFDPW	1938	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-D10	LRP6e3e4	YIFTDYYMH	1669	GWMNPNSGNTGYA	1776	CARTLSGYSSSWYVFDYW	1964	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-E10	LRP6e3e4	FTFSSYSMN	1579	SGISWNSGTTGYS	1797	CARDHSSGWRHYFDYW	1895	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-F10	LRP6e3e4	FTFSNSDMN	1570	SYISGNSGYTNYA	1814	CASGSYYSDFDYW	1971	RASQSISNYLN	2090	AASTLES	2111	CQQANSFPPTF	2148
010S-G10	LRP6e3e4	GTFSSYAIS	1603	GRINPNGGGTIYA	1769	CAREGGYYFDYW	1919	RASQGISNYLA	2083	AASSLQS	2110	CQQSYSTPWTF	2175
009S-F07	LRP6e3e4	GTFSSYAIS	1603	GIINPSGGSTSYA	1761	CARAAGNFWSGYYTFDYW	1877	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-G07	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CARGSYGMDVW	1948	RASQGISNYLA	2083	DASNLET	2115	CLQDFSFPWTF	2140
010S-H10	LRP6e3e4	YTFTSYYMH	1680	GWINPNSGGTNYA	1774	CAREAAEIPVGAFDIW	1914	KSSHSLLYSSDNKNYLA	2071	WSSTRES	2136	CQQYYSTPQTF	2188
010S-A11	LRP6e3e4	FTFSNSDMN	1570	SYISGNSGYTNYA	1814	CASGSYYSDFDYW	1972	RASQSIZNYLN	2095	ZASTLES	2138	CQQANSFPPTF	2148
010S-B11	LRP6e3e4	FTFRNYAIH	1564	SAIGTGGDTYYA	1780	CARDGGIRDFDYW	1894	QASQDISNYLN	2077	AASTLQS	2113	CQQSYSTPLTF	2174
010S-C11	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CAADDLGLELHYW	1822	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-H07	LRP6e3e4	YTFTGYYMH	1675	GWMNPNSGNTGYA	1776	CASSVVPAGPAGVYAFDIW	1976	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
009S-A08	LRP6e3e4	GTFSSHAIN	1602	GWISANNGNTDYA	1775	CARDQDYGDYGWYYYGMDVW	1903	RASQGISNYLA	2083	GSSTLQS	2127	CQQTYSIPPTF	2181
010S-D11	LRP6e3e4	YTFTYRYLH	1681	GGIIPVFGTANYA	1752	CATDEYSSSYAFDIW	1983	RASQSVSSNLA	2097	GASTRAT	2125	CQQFDRSPLTF	2151
010S-E11	LRP6e3e4	FTFSAHGMH	1565	SGISESGGSTYYA	1791	CARGRGYSYGYYAFDIW	1943	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-F11	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CARDSDWGVVDPW	1905	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-G11	LRP6e3e4	YTFTYRYLH	1681	GRIIPVLKITNYA	1768	CAVVDDAFDIW	1997	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-H11	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CAKDGTDGRFDPW	1846	RASQDISSYLA	2080	SASTLQS	2133	CQQSNSFPYTF	2157
009S-B08	LRP6e3e4	FTFTSSAVQ	1589	GWINAGNGNTTYA	1772	CARRGGDVTVPAAYYAMDVW	1963	RASQSISSYLN	2094	ZASSLQS	2137	CQQSYSTPLTF	2174
010S-A12	LRP6e3e4	VTFSRYPIS	1667	GGIIPIFGTANYA	1749	CAKDSGNYGYYGMDVW	1854	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-B12	LRP6e3e4	FTFSSYDMH	1578	SGITSNGGATYYA	1798	CARGTTGKGYYYYGMDVW	1949	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-C12	LRP6e3e4	FTFSNYWIH	1571	SAIGTGGGTYYA	1781	CTTAGYKAARRSVYPRIFNFDYW	2044	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-D12	LRP6e3e4	YTFTYRYLH	1681	GRIIPIFGTANYA	1763	CAREEGVGGMDVW	1917	RPSQSIGSWLA	2100	DASNLQS	2116	CQQSSSTPYTF	2158
010S-E12	LRP6e3e4	FTFSSYAMH	1576	SAIGAGGGTYYA	1779	CARGVSSGYYYYYGMDVW	1950	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
010S-F12	LRP6e3e4	FTVSSNYMS	1592	SAIGTGGGTYYA	1781	CARAGTNWGGWYFDLW	1879	RASQGISRDLA	2085	AASTLQS	2113	CQQSYSPPFTF	2172
010S-G12	LRP6e3e4	FALSGYYMS	1550	SSISSSSTYIRYA	1803	CATVTGYSSAGAFDIW	1995	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-A01	LRP6e3e4	FTFSTHAFH	1583	SAIRGSGERTYYA	1784	CARDLRNWGSPYWYFDLW	1901	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-B01	LRP6e3e4	GTFSHYTIS	1600	GWINAGNGNTKYS	1771	CAKGGSLDMDVW	1864	RASQGISNYLA	2083	AASSLHS	2108	CQQSYRTPLTF	2164
011S-C01	LRP6e3e4	LTFTSHGMS	1615	SYVSDSGSSVYYA	1818	CARHPGSFGGYSYAWYYYYGMDVW	1957	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
011S-D01	LRP6e3e4	GTISDYTVS	1605	GIINPSGGSTSYA	1761	CARGYYDFDYW	1953	RASQGISNYLA	2083	AASSLQS	2110	CQQSYSTPLTF	2174
009S-C08	LRP6e3e4	FSFNTFGIH	1556	AVISYDGSNKYYA	1738	CAKSIAAAGTGYYGMDVW	1869	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-E01	LRP6e3e4	FPFZYYSMN	1552	SAISGRDGRTYYA	1785	CAKDLGIQLPDYYFDYW	1847	RASQGISSALA	2086	AASTLQS	2113	CQQSYSSPPTF	2173
009S-D08	LRP6e3e4	YTFTSYDIN	1679	GGIIPIFGTANYA	1749	CARGPYYFDYW	1941	RASQGISNNLN	2082	DASSLES	2120	CLQHNSYPFTF	2143
011S-F01	LRP6e3e4	FSFSDYYMS	1558	SGISESGGRTYYA	1790	CASAADFDYW	1967	RASQDISNYLN	2079	AASSLQS	2110	CLQDYSYPRTF	2141
009S-E08	LRP6e3e4	YGFTGYYIH	1668	GWMNPNSGNTGYA	1776	CARGYGDYDLW	1952	QASQDISNYLN	2077	DASSLES	2120	CQQSYRYPTF	2165
009S-F08	LRP6e3e4	DTFANYGFS	1547	GXVNAGNGNTTYA	1777	CAKGWLDFDYW	1867	QASQDISNYLN	2077	DASSLES	2120	CQQSYSTSITF	2177
011S-G01	LRP6e3e4	YTFTYRYLH	1681	GGIIPLFGTANYA	1750	CTTDDYGDQYGMDVW	2046	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-H01	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CTTDDYGDLTHLDYW	2045	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-A02	LRP6e3e4	GTFSSYAIS	1603	GWMNPNSGNTGYA	1776	CARDKGYAFDIW	1896	RSSQSLLHSNGYNYLD	2102	AASSLQS	2110	CMQALQTPITF	2145
011S-B02	LRP6e3e4	YSFTRTDMH	1670	GYISAYTGHTSYA	1778	CARDLGGTADYW	1899	RZSQSZSZYLN	2104	AASSLQS	2110	CQQSYSTPLTF	2174
011S-C02	LRP6e3e4	FTFSTYSMN	1585	SGISWNSGRIGYA	1795	CARDVGAFDIW	1906	QASQDISNYLN	2077	AASILQS	2107	CQQSYSIPFTF	2169
009S-G08	LRP6e3e4	FTFSDFAMT	1566	SYISGDSGYTNYA	1813	CARLGSYPGPYYYYMDVW	1962	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-D02	LRP6e3e4	FTFSSYAMS	1577	SSISGSGGVTYYA	1801	CARGGNTYYYYYGMDVW	1928	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-H08	LRP6e3e4	YTFTDYFMN	1673	GIINPSGDSTRFA	1758	CARDDGLGGMDVW	1889	QASQDISNYLA	2076	AASSLQS	2110	CQQSYSTPLTF	2174
011S-E02	LRP6e3e4	YTFTYRYLH	1681	GGIIPIFGTANYA	1749	CATDYGDYYYGMDVW	1984	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-A09	LRP6e3e4	YTFTYRYLH	1681	GRIIPILGSTNYA	1767	CTTDLWDYW	2048	QASQGITNYLN	2078	AASSLQS	2110	CLQDYTDPFTF	2142

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
011S-F02	LRP6e3e4	FTFSTYGMH	1584	SSISVSSGTTHYA	1805	CARGGSGSYYYAFDIW	1930	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-G02	LRP6e3e4	YTFTSYAMN	1678	GGIIPIFGTANYA	1749	CARDASGGSTGWYYFDSW	1887	RASQGISSYLA	2087	AASSLQS	2110	CQQAYSFPWTF	2150
011S-H02	LRP6e3e4	YTFTNNFMH	1676	GIINPSGGSTSYA	1761	CARGLYKRYSYGYGMDVW	1935	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
009S-B09	LRP6e3e4	FSFNTYAMN	1557	AVTSYDGGKKNYA	1742	CARDAGGDYDYW	1885	QASQDISNYLN	2077	AASSLQS	2110	CQQSYNTPRTF	2161
009S-C09	LRP6e3e4	GTFHTYGLS	1597	GGIIPIFGTANYA	1749	CARGSGWSGLDYW	1945	QASQDISNYLN	2077	DASNLET	2115	CQQSYTTPFTF	2180
011S-A03	LRP6e3e4	FTFSSYWMH	1580	STISGSGGRTYYA	1808	CATSPYGVFTLDYW	1994	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-B03	LRP6e3e4	GTFSZYAIS	1604	GIINPSGGSTNYA	1760	CARAGYWSGYGYYGMDVW	1881	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-C03	LRP6e3e4	YTFSYRYLH	1672	GGIIPIFGTANYA	1749	CASTVTTDAFDIW	1978	QASQDISNYLN	2077	DASSLES	2120	CQQSYSFPPFTF	2168
011S-D03	LRP6e3e4	FSFDDYGMS	1554	SVISSGGTIYYA	1812	CARHLSSGYLSYYGMDVW	1955	RASQSISSYLA	2093	AASTLQS	2113	CQQSYSTPLTF	2174
009S-F09	LRP6e3e4	YSFTRTDMH	1670	GYISAYTGHTSYA	1778	CARDLGGTADYW	1900	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-E03	LRP6e3e4	FTFSSYAMS	1577	SAISGSGGSTYYA	1786	CAKGGRDGYKGYFDYW	1861	KSSHSLLSTSTNRNQLA	2070	WASSRKS	2135	CQQYNNWPYTF	2186
009S-G09	LRP6e3e4	FTFSRHSMN	1572	SYSSGNSGYTNYA	1817	CARGDLEFDYW	1923	RASQGISNYLA	2083	SASSLQS	2132	CQQGYNTPRTF	2154
011S-F03	LRP6e3e4	FTFSSYAMS	1577	SAISGSGGSTYYA	1786	CAKGGRDGYKGYFDYW	1862	KSSQSVLYTTTNRNHIA	2074	WASSRKS	2135	CQQYYSTPYTF	2189
009S-H09	LRP6e3e4	FTFSSYAMS	1577	SAISGSGGSTYYA	1786	CAKGGRDGYKGYFDYW	1863	KSSHSLLSTSTNRNHLA	2069	WASSRKS	2135	CQQYYNIPYSF	2187
011S-G03	LRP6e3e4	YTFTYRYLH	1681	GRIIPIHGIANYA	1764	CAREYSYGYFRYW	1922	RASQGISSYLA	2087	DASNLET	2115	CQQANSLFTF	2149
009S-A10	LRP6e3e4	FTFTSSAMQ	1588	GIINPSGGSTIYA	1759	CASGDTYDLYSLDVW	1970	RASQSISRWLA	2091	AASSLQS	2110	CQQAYSFPWTF	2150
009S-B10	LRP6e3e4	YIFTDYYMH	1669	GWINAGNGNTTYA	1772	CAKVASGWSWPFDIW	1871	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174

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Clone ID	Confirmed Binding	CDRH1	CDRH1 SEQ ID	CDRH2	CDRH2 SEQ ID	CDRH3	CDRH3 SEQ ID	CDRL1	CDRL1 SEQ ID	CDRL2	CDRL2 SEQ ID	CDRL3	CDRL3 SEQ ID
011S-B04	LRP6e1e2	YTFTSYDIN	1679	GIINPSGGSTSYA	1761	CTREHSYYYYGMDVW	2042	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-C04	LRP6e1e2	GTFNSNAIS	1598	GWMNPNSGNTGYA	1776	CARDYYGSGSYNYGMDVW	1913	GASQSVPRNSLA	2066	GASQRAT	2124	CQQYHNWPPEYTF	2184
011S-D04	LRP6e1e2	YTFTSYDIN	1679	GIINPSGGSTSYA	1761	CAREAYYYYYGMDVW	1916	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-E04	LRP6e1e2	FTFSSZZMH	1581	SAIGTGGGTZYA	1782	CAKDLGRAAAGSMDVW	1850	WASQSVRGNYVA	2106	DASNRAA	2117	CQHRSNWPLTF	2147
011S-F04	LRP6e1e2	YIFTDYYMH	1669	GRIIPILGRANYA	1765	CARGGYSTLDYW	1933	HGSQDISNYLN	2067	DASNRQS	2119	CQQSFSTPRTF	2156
011S-H04	LRP6e1e2	YIFTDYYMH	1669	GRIIPILGRANYA	1765	CARGGYSTLDYW	1934	QASQDISNYLN	2077	AASTLQS	2113	CQQSFSTPRTF	2156
011S-A05	LRP6e1e2	FTFSSYAMH	1576	SAIGTGGGTYYA	1781	CAKDLGRAAAGSMDVW	1848	WASQSVRGNYVA	2106	DASNRAG	2118	CQHRSNWPLTF	2147
011S-B05	LRP6e1e2	YZFTDYYMH	1683	GWMNPNSGNTGYA	1776	CTRVAWGLDYW	2043	RASQSISSYLN	2094	AASSLQS	2110	CQQSYSTPLTF	2174
011S-C05	LRP6e1e2	FTFSSYAMH	1576	SAIGTGGGTYYA	1781	CAKDLGRAAAGSMDVW	1849	WASQSVRGNYVA	2106	DASNRAA	2117	CQHRSNWPLTF	2147
1115.3	LRP6e1e2	GFSFSTS		NLNGGS		ELAGYGTPFAY		KASQSISYNLH		YTSQSIS		QQSNSWPLT	
421.1	LRP6e1e2	GYTFTTY		FPGNVNT		EELQYYFDY		SANSSVRFMF		RTSNLAS		QQYHSYPWT	
YW211.31.57	LRP6e3e4	GFTFTSY		SPYSGS		RARPPIRLHPRGSVMDY		RASQDVSTAVA		SASFLYS		QQSYTTPPT	

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Table 2B. Anti-LRP5/6 Antibody Clone IDs, Heavy Chain (HC) Seq ID Nos, and Binding Characteristics.

Clone ID	HC Seq ID NO	Confirmed Binding
001S-F11	66	LRP6e1e2
009S-G02	67	LRP6e1e2
009S-A03	68	LRP6e1e2
009S-D03	69	LRP6e1e2
009S-F03	70	LRP6e1e2
009S-H03	71	LRP6e1e2
009S-A04	72	LRP6e1e2
009S-B04	73	LRP6e3e4
009S-D04	74	LRP6e3e4
009S-E04	75	LRP6e3e4
009S-F04	76	LRP6e3e4
009S-G04	77	LRP6e3e4
009S-H04	78	LRP6e3e4
009S-A05	79	LRP6e3e4
013S-G04	80	LRP6e3e4
013S-H04	81	LRP6e3e4
013S-C05	82	LRP6e3e4
013S-D05	83	LRP6e3e4
013S-G04	84	LRP6e3e4
013S-H04	85	LRP6e3e4
013S-A05	86	LRP6e3e4
013S-C05	87	LRP6e3e4
013S-D05	88	LRP6e3e4
008S-D01	133	LRP5

In certain embodiment, the LRP5/6 binding domain may be selected from any binding domain that binds LRP5 or LRP6 with a K_D of less than or equal to about 1 x 10^{-4} M, less than or equal to about 1 x 10^{-5} M, less than or equal to about 1 x 10^{-6} M, less than or equal to about 1 x 10^{-7} M, less than or equal to about 1 x 10^{-8} M, less than or equal to about 1 x 10^{-9} M, or less than or equal to about 1 x 10^{-10} M in the context of a Wnt surrogate molecule. In certain embodiment, the LRP5/6 binding domain may be selected from any binding domain that binds LRP5 or LRP6 with a K_D of greater than or equal to about 1 x 10^{-4} M, greater than or equal to about 1 x 10^{-5} M, greater than or equal to about 1 x 10^{-6} M, greater than or equal to about 1 x 10^{-7} M, greater than or equal to about 1 x 10^{-8} M, greater than or equal to about 1 x 10^{-9} M, or greater than about 1 x 10^{-10} M in the context of a Wnt surrogate molecule. In certain embodiment, the LRP5/6 binding domain may be selected from any binding domain that binds LRP5 or LRP6 at high affinity, e.g. a K_D of less than about 1 x 10^{-7} M, less than about 1 x 10^{-8} M, less than about 1 x 10^{-9} M, or less than about 1 x 10^{-10} M.

Other suitable LRP5/6 binding domains include, without limitation, de novo designed LRP5/6 binding proteins, antibody derived binding proteins, *e.g.* scFv, Fab, *etc.* and other portions of antibodies that specifically bind to one or more Fzd proteins; VHH or sdAb derived binding domains; knottin-based engineered scaffolds; naturally occurring LRP5/6, including without limitation, DKK1, DKK2, DKK3, DKK4, sclerostin; Wise; fusions proteins comprising any of the above; derivatives of any of the above; variants of any of the above; and biologically active fragments of any of the above, and the like. A LRP5/6 binding domain may be affinity selected to enhance binding.

Members of the Dickkopf (DKK) gene family (see Krupnik et al. (1999) Gene 238(2):301-13) include DKK-1, DKK-2, DKK-3, and DKK-4, and the DKK-3 related protein Soggy (Sgy). hDKKs 1-4 contain two distinct cysteine-rich domains in which the positions of 10 cysteine residues are highly conserved between family members. Exemplary sequences of human Dkk genes and proteins are publicly available, e.g., Genbank accession number NM_014419

(soggy-1); NM_014420 (DKK4); AF177394 (DKK-1); AF177395 (DKK-2); NM_015881 (DKK3); and NM_014421 (DKK2). In some embodiments of the invention, the Lrp6 binding moiety is a DKK1 peptide, including without limitation the C-terminal domain of human DKK1. The C-terminal domain may comprise the sequence:
KMYHTKGQEGSVCLRSSDCASGLCCARHFWSKICKPVLKEGQVCTKHRRKGS HGLEIFQRCYCGEGLSCRIQKDHHQASNSSRLHTCQRH (SEQ ID NO:2190) (see Genbank accession number NP_036374) or a biologically active fragment thereof.

Binding of DKK proteins to LRP5/6 are discussed, for example in Brott and Sokol Mol. Cell. Biol. 22 (17), 6100-6110 (2002); and Li et al. J. Biol. Chem. 277 (8), 5977-5981 (2002), each herein specifically incorporated by reference. The corresponding region of human DKK2 (Genbank reference NP_055236) may comprise the sequence:
KMSHIKGHEGDPCLRSSDCIEGFCCARHFWTKICKPVLHQGEVCTKQRKKGSH GLEIFQRCDCAKGLSCKVWKDATYSSKARLHVCQK (SEQ ID NO:2191) or a biologically active fragment thereof.

Antibodies that specifically bind to LRP5 or LRP6 are known in the art and are commercially available, or can be generated de novo. LRP5, LRP6 or fragments thereof can be used as an immunogen or in screening assays to develop an antibody. Examples of known antibodies include, without limitation, those described in Gong et al. (2010) PLoS One. 5(9):e12682; Ettenberg et al. (2010) Proc Natl Acad Sci U S A. 107(35):15473-8; and those commercially available from, for example Santa Cruz biotechnology antibody clone 1A12, which was raised against synthetic LRP5/6 of human origin and binds to both the full length and proteolytic fragment of LRP6 and LRP5 of mouse and human origin; the monoclonal antibody 2B11; Cell Signaling Technology antibody specific for LRP5 (D80F2), catalog number 5731; etc.

In certain embodiments, Wnt surrogate molecules disclosed herein comprise one or more polypeptides comprising two or more binding regions. For example, the two or more binding regions may be two or more Fzd binding

regions or two or more LRP5/6 binding regions, or they may comprise one or more Fzd binding region and one or more LRP5/6 binding region. The binding regions may be directly joined or contiguous, or may be separated by a linker, e.g. a polypeptide linker, or a non-peptidic linker, etc. The length of the linker, and therefore the spacing between the binding domains can be used to modulate the signal strength, and can be selected depending on the desired use of the Wnt surrogate molecule. The enforced distance between binding domains can vary, but in certain embodiments may be less than about 100 angstroms, less than about 90 angstroms, less than about 80 angstroms, less than about 70 angstroms, less than about 60 angstroms, or less than about 50 angstroms. In some embodiments the linker is a rigid linker, in other embodiments the linker is a flexible linker. In certain embodiments where the linker is a peptide linker, it may be from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acids in length, and is of sufficient length and amino acid composition to enforce the distance between binding domains. In some embodiments, the linker comprises or consists of one or more glycine and/or serine residues.

In particular embodiments, a Wnt surrogate molecule comprises a polypeptide sequence having at least 90%, at least 95%, at least 98% or at least 99% identity to a polypeptide sequence disclosed in any of SEQ ID NOs:89-128 or 134-157, or having at least 90%, at least 95%, at least 98% or at least 99% identity to an antigen-binding fragment of a polypeptide sequence disclosed in any of SEQ ID NOs:89-128 or 134-157. In certain embodiments, the Wnt surrogate molecules comprises or consists of a polypeptide sequence set forth in any of SEQ ID NOs:89-128 or 134-157, or an antigen-binding fragment thereof. In particular embodiments, the antigen-binding fragment binds one or more Fzd receptors and also binds LRP5 and/or LRP6.

Wnt surrogate molecule can be multimerized, e.g. through an Fc domain, by concatenation, coiled coils, polypeptide zippers, biotin/avidin or streptavidin multimerization, and the like. The Wnt surrogate molecules can also

be joined to a moiety such as PEG, Fc, etc., as known in the art to enhance stability in vivo.

In certain embodiments, a Wnt surrogate molecule directly activates canonical Wnt signaling through binding to one or more Fzd proteins and to LRP5/6, particularly by binding to these proteins on a cell surface, e.g., the surface of a human cell. The direct activation of Wnt signaling by a Wnt surrogate molecule is in contrast to potentiation of Wnt signaling, which enhances activity only when native Wnt proteins are present.

Wnt surrogate molecules may activate Wnt signaling, e.g., by mimicking the effect or activity of a Wnt protein binding to a frizzled protein. The ability of the Wnt surrogate molecules of the invention to mimic the activity of Wnt can be confirmed by a number of assays. The Wnt surrogate molecules typically initiate a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand. In particular, the Wnt surrogate molecules of the invention enhance the canonical Wnt/β-catenin signaling pathway. As used herein, the term "enhances" refers to a measurable increase in the level of Wnt/β-catenin signaling compared with the level in the absence of a Wnt surrogate molecule of the invention.

Various methods are known in the art for measuring the level of canonical Wnt/β-catenin signaling. These include, but are not limited to assays that measure: Wnt/β-catenin target gene expression; TCF reporter gene expression; β-catenin stabilization; LRP phosphorylation; Axin translocation from cytoplasm to cell membrane and binding to LRP. The canonical Wnt/β-catenin signaling pathway ultimately leads to changes in gene expression through the transcription factors TCF7, TCF7L1, TCF7L2 (a.k.a. TCF4), and LEF. The transcriptional response to Wnt activation has been characterized in a number of cells and tissues. As such, global transcriptional profiling by methods well known in the art can be used to assess Wnt/β-catenin signaling activation or inhibition.

Changes in Wnt-responsive gene expression are generally mediated by TCF and LEF transcription factors. A TCF reporter assay assesses changes in the transcription of TCF/LEF controlled genes to determine the level

of Wnt/β-catenin signaling. A TCF reporter assay was first described by Korinek, V. et al., 1997. Also known as TOP/FOP this method involves the use of three copies of the optimal TCF motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal c-Fos promoter driving luciferase expression (pTOPFl_ASH and pFOPFl_ASH, respectively) to determine the transactivational activity of endogenous p-catenin/TCF4. A higher ratio of these two reporter activities (TOP/FOP) indicates higher β-catenin/TCF4 activity, whereas a lower ratio of these two reporter activities indicates lower β-catenin/TCF4 activity.

Various other reporter transgenes that respond to Wnt signals exist intact in animals and therefore, effectively reflect endogenous Wnt signaling. These reporters are based on a multimerized TCF binding site, which drives expression of LacZ or GFP, which are readily detectable by methods known in the art. These reporter genes include: TOP-GAL, BAT-GAL, ins-TOPEGFP, ins-TOPGAL, LEF-EGFP, Axin2-LacZ, Axin2-d2EGFP, Lgr5tm1 (cre/ERT2), TOPdGFP.

The recruitment of dephosphorylated β-catenin to the membrane, stabilization and phosphorylation status of β-catenin, and translocation of β-catenin to the nucleus (Klapholz- Brown Z et al., PLoS One. 2(9) e945, 2007), in some cases mediated by complex formation with TCF transcription factors and TNIK are key steps in the Wnt signaling pathway. Stabilization is mediated by Disheveled family proteins that inhibit the "destruction" complex so that degradation of intracellular β-catenin is reduced, and translocation of β-catenin to the nucleus follows thereafter. Therefore, measuring the level and location of β-catenin in a cell is a good reflection of the level of Wnt/β-catenin signaling. A non-limiting example of such an assay is the "BioImage β-Catenin Redistribution Assay" (Thermo Scientific) which provides recombinant U20S cells that stably express human β-catenin fused to the C-terminus of enhanced green fluorescent protein (EGFP). Imaging and analysis is performed with a fluorescence microscope or HCS platform allowing the levels and distribution of EGFP-β-catenin to be visualized.

Another way, in which the destruction complex is inhibited, is by removal of Axin by recruitment of Axin to the cytoplasmic tail of the Wnt co-receptor LRP. Axin has been shown to bind preferentially to a phosphorylated form of the LRP tail. Visualization of Axin translocation, for example with a GFP-Axin fusion protein, is therefore another method for assessing levels of Wnt/β-catenin signaling.

In certain embodiments, a Wnt surrogate molecule enhances or increases canonical Wnt pathway signaling, e.g., β-catenin signaling, by at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 150%, 200%, 250%, 300%, 400% or 500%, as compared to the β-catenin signaling induced by a neutral substance or negative control as measured in an assay described above, for example as measured in the TOPFlash assay. A negative control may be included in these assays. In particular embodiments, Wnt surrogate molecules may enhance β-catenin signaling by a factor of 2x, 5x, 10x, 100x, 1000x, 10000x or more as compared to the activity in the absence of the Wnt surrogate molecule when measured in an assay described above, for example when measured in the TOPFlash assay, or any of the other assays mentioned herein.

"Wnt gene product" or "Wnt polypeptide" when used herein encompass native sequence Wnt polypeptides, Wnt polypeptide variants, Wnt polypeptide fragments and chimeric Wnt polypeptides. In particular embodiments, a Wnt polypeptide is a native human full length mature Wnt protein.

For example, human native sequence Wnt proteins of interest in the present application include the following: Wnt-1 (GenBank Accession No. NM_005430); Wnt-2 (GenBank Accession No. NM_003391); Wnt-2B (Wnt-13) (GenBank Accession No. NM_004185 (isoform 1), NM_024494.2 (isoform 2)), Wnt-3 (RefSeq.: NM_030753), Wnt3a (GenBank Accession No. NM_033131), Wnt-4 (GenBank Accession No. NM_030761), Wnt-5A (GenBank Accession No. NM_003392), Wnt-5B (GenBank Accession No. NM_032642), Wnt-6 (GenBank Accession No. NM_006522), Wnt-7A (GenBank Accession No. NM_004625),

Wnt- 7B (GenBank Accession No. NM_058238), Wnt-8A (GenBank Accession No. NM_058244), Wnt-8B (GenBank Accession No. NM_003393), Wnt-9A (Wnt-14) (GenBank Accession No. NM_003395), Wnt-9B (Wnt-15) (GenBank Accession No. NM_003396), Wnt-1 OA (GenBank Accession No. NM_025216), Wnt-10B (GenBank Accession No. NM_003394), Wnt-11 (GenBank Accession No. NM_004626), Wnt- 16 (GenBank Accession No. NM_016087)). Although each member has varying degrees of sequence identity with the family, all encode small (i.e., 39-46 kD), acylated, palmitoylated, secreted glycoproteins that contain 23-24 conserved cysteine residues whose spacing is highly conserved (McMahon, A P et al., Trends Genet. 1992; 8: 236-242; Miller, J R. Genome Biol. 2002; 3(1): 3001.1-3001.15). Other native sequence Wnt polypeptides of interest include orthologs of the above from any mammal, including domestic and farm animals, and zoo, laboratory or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, rats, mice, frogs, zebra fish, fruit fly, worm, etc.

"Wnt pathway signaling" or "Wnt signaling" is used herein to refer to the mechanism by which a biologically active Wnt exerts its effects upon a cell to modulate a cell's activity. Wnt proteins modulate cell activity by binding to Wnt receptors, including proteins from the Frizzled (Fzd) family of proteins, proteins from the ROR family of proteins, the proteins LRP5, LRP6 from the LRP family of proteins, the protein FRL1/crypto, and the protein Derailed/Ryk. Once activated by Wnt binding, the Wnt receptor(s) will activate one or more intracellular signaling cascades. These include the canonical Wnt signaling pathway; the Wnt/planar cell polarity (Wnt/PCP) pathway; the Wnt-calcium (Wnt/Ca^{2+}) pathway (Giles, RH et al. (2003) Biochim Biophys Acta 1653, 1-24; Peifer, M. et al. (1994) Development 120: 369-380; Papkoff, J. et al (1996) Mol. Cell Biol. 16: 2128-2134; Veeman, M. T. et al. (2003) Dev. Cell 5: 367-377); and other Wnt signaling pathways as is well known in the art.

For example, activation of the canonical Wnt signaling pathway results in the inhibition of phosphorylation of the intracellular protein β-catenin, leading to an accumulation of β- catenin in the cytosol and its subsequent

translocation to the nucleus where it interacts with transcription factors, e.g. TCF/LEF, to activate target genes. Activation of the Wnt/PCP pathway activates RhoA, c-Jun N-terminal kinase (JNK), and nemo-like kinase (NLK) signaling cascades to control such biological processes as tissue polarity and cell movement. Activation of the Wnt/Ca^{2+} by, for example, binding of Wnt-4, Wnt-5A or Wnt-11, elicits an intracellular release of calcium ions, which activates calcium sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII) or calcineurin (CaCN). By assaying for activity of the above signaling pathways, the biological activity of an antibody or antigen-binding fragment thereof, e.g., a Wnt surrogate molecule, can be readily determined.

In certain embodiments, functional properties of Wnt surrogate molecules may be assessed using a variety of methods known to the skilled person, including e.g., affinity/binding assays (for example, surface plasmon resonance, competitive inhibition assays), cytotoxicity assays, cell viability assays, cell proliferation or differentiation assays in response to a Wnt, cancer cell and/or tumor growth inhibition using *in vitro* or *in vivo* models, including but not limited to any described herein. The Wnt surrogate molecules described herein may also be tested for effects on Fzd receptor internalization, *in vitro* and *in vivo* efficacy, *etc*. Such assays may be performed using well-established protocols known to the skilled person (see *e.g.*, Current Protocols in Molecular Biology (Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., NY, NY); Current Protocols in Immunology (Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober 2001 John Wiley & Sons, NY, NY); or commercially available kits.

In certain embodiments, a Fzd-binding region of a Wnt surrogate molecule (e.g., an antigen-binding fragment of an anti-Fzd antibody) comprises one or more of the CDRs of the anti-Fzd antibodies described herein. In certain embodiments, a LRP5/6-binding region of a Wnt surrogate molecule (e.g., an antigen-binding fragment of an anti-LRP5/6 antibody) comprises one or more of the CDRs of the anti-LRP5/6 antibodies described herein. In this regard, it has been shown in some cases that the transfer of only the VHCDR3 of an antibody

can be performed while still retaining desired specific binding (Barbas *et al.*, *PNAS* (1995) 92: 2529-2533). See also, McLane *et al.*, *PNAS* (1995) 92:5214-5218, Barbas *et al.*, *J. Am. Chem. Soc.* (1994) 116:2161-2162.

Also disclosed herein is a method for obtaining an antibody or antigen binding domain specific for a Fzd receptor, the method comprising providing by way of addition, deletion, substitution or insertion of one or more amino acids in the amino acid sequence of a VH domain set out herein or a VH domain which is an amino acid sequence variant of the VH domain, optionally combining the VH domain thus provided with one or more VL domains, and testing the VH domain or VH/VL combination or combinations to identify a specific binding member or an antibody antigen binding domain specific for one or more Fzd receptor and optionally with one or more desired properties. The VL domains may have an amino acid sequence which is substantially as set out herein. An analogous method may be employed in which one or more sequence variants of a VL domain disclosed herein are combined with one or more VH domains.

In particular embodiments, Wnt surrogate molecules are water soluble. By "water soluble" it is meant a composition that is soluble in aqueous buffers in the absence of detergent, usually soluble at a concentration that provides a biologically effective dose of the polypeptide. Compositions that are water soluble form a substantially homogenous composition that has a specific activity that is at least about 5% that of the starting material from which it was purified, usually at least about 10%, 20%, or 30% that of the starting material, more usually about 40%, 50%, or 60% that of the starting material, and may be about 50%, about 90% or greater. Wnt surrogate molecules disclosed herein typically form a substantially homogeneous aqueous solution at concentrations of at least 25 µM and higher, e.g. at least 25 µM, 40 µM, or 50 µM, usually at least 60 µM, 70 µM, 80 µM, or 90 µM, sometimes as much as 100 µM, 120 µM, or 150 µM. In other words, Wnt surrogate molecules disclosed herein typically form a substantially homogeneous aqueous solution at concentrations of about 0.1 mg/ml, about 0.5 mg/ml, of about 1 mg/ml or more.

An antigen or epitope that "specifically binds" or "preferentially binds" (used interchangeably herein) to an antibody or antigen-binding fragment thereof is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule, e.g., a Wnt surrogate molecule, is said to exhibit "specific binding" or "preferential binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. A molecule or binding region thereof, e.g., a Wnt surrogate molecule or binding region thereof, "specifically binds" or "preferentially binds" to a target antigen, e.g., a Fzd receptor, if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, a Wnt surrogate molecule or binding region thereof that specifically or preferentially binds to the Fzd1 receptor is an antibody that binds to the Fzd1 receptor with greater affinity, avidity, more readily, and/or with greater duration than it binds to other Fzd receptors or non-Fzd proteins. It is also understood by reading this definition that, for example, a Wnt surrogate molecule or binding region thereof that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

In some embodiments, any of the one or more Fzd binding region of a Wnt surrogate molecule binds to one, two, three, four, five or more different frizzled proteins, e.g., one or more of human frizzled proteins Fzd1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7, Fzd8, Fzd9, Fzd10. In some embodiments, any of the Fzd binding regions binds to Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8. In various embodiments, any of the Fzd binding regions binds to: (i) Fzd1, Fzd2, Fzd7 and Fzd9; (ii) Fzd1, Fzd2 and Fzd7; (iii) Fzd5 and Fzd8; (iv) Fzd5, Fzd7 and Fzd8; (v) Fzd1, Fzd4, Fzd5 and Fzd8; (vi) Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8; (vii) Fzd4 and Fzd9; (viii) Fzd9 and Fzd10; (ix) Fzd5, Fzd8 and Fzd10; or (x) Fzd4, Fzd5 and Fzd8; Fzd1, Fzd5, Fzd7 and Fzd8. In some embodiments, the Fzd binding

region is selective for one or more Fzd protein of interest, e.g. having a specificity for the one or more desired Fzd protein of at least 10-fold, 25-fold, 50-fold, 100-fold, 200-fold or more relative to other Fzd proteins. In some embodiments, any of the one or more Fzd binding region of a Wnt surrogate molecule is monospecific and binds or specifically binds to only one of Fzd1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7, Fzd8, Fzd9, or Fzd10.

In some embodiments, any of the one or more LRP5/6 binding region of a Wnt surrogate molecule binds to one or both of LRP5/6. For convenience, the term “LRP5/6” is used to refer collectively to either or both of LRP5 and/or LRP6.

Immunological binding generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific, for example by way of illustration and not limitation, as a result of electrostatic, ionic, hydrophilic and/or hydrophobic attractions or repulsion, steric forces, hydrogen bonding, van der Waals forces, and other interactions. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off} /K_{on} enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant K_d. See, generally, Davies *et al.* (1990) *Annual Rev. Biochem. 59*:439-473.

In certain embodiments, the Wnt surrogate molecules or binding regions thereof described herein have an affinity of less than about 10,000, less

than about 1000, less than about 100, less than about 10, less than about 1 or less than about 0.1 nM, and in some embodiments, the antibodies may have even higher affinity for one or more Fzd receptor or LRP5 or LRP6 receptor.

The constant regions of immunoglobulins show less sequence diversity than the variable regions, and are responsible for binding a number of natural proteins to elicit important biochemical events. In humans, there are five different classes of antibodies including IgA (which includes subclasses IgA1 and IgA2), IgD, IgE, IgG (which includes subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. The distinguishing features between these antibody classes are their constant regions, although subtler differences may exist in the V region.

The Fc region of an antibody interacts with a number of Fc receptors and ligands, imparting an array of important functional capabilities referred to as effector functions. For IgG, the Fc region comprises Ig domains CH2 and CH3 and the N-terminal hinge leading into CH2. An important family of Fc receptors for the IgG class are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system (Raghavan *et al.*, 1996, Annu Rev Cell Dev Biol 12:181-220; Ravetch *et al.*, 2001, Annu Rev Immunol 19:275-290). In humans this protein family includes FcγRI (CD64), including isoforms FcγRIa, FcγRIb, and FcγRIc; FcγRII (CD32), including isoforms FcγRIIa (including allotypes H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16), including isoforms FcγRIIIa (including allotypes V158 and F158) and FcγRIIIb (including allotypes FcγRIIIb-NA1 and FcγRIIIb-NA2) (Jefferis *et al.*, 2002, Immunol Lett 82:57-65). These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling event within the cell. These receptors are expressed in a variety of immune cells including monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, B cells, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, and T cells. Formation of the Fc/FcγR complex recruits these effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important

subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack.

The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) (Raghavan *et al.*, 1996, Annu Rev Cell Dev Biol 12:181-220; Ghetie *et al.*, 2000, Annu Rev Immunol 18:739-766; Ravetch *et al.*, 2001, Annu Rev Immunol 19:275-290). The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell is referred to as antibody dependent cell-mediated phagocytosis (ADCP). All FcγRs bind the same region on Fc, at the N-terminal end of the Cg2 (CH2) domain and the preceding hinge. This interaction is well characterized structurally (Sondermann *et al.*, 2001, J Mol Biol 309:737-749), and several structures of the human Fc bound to the extracellular domain of human FcγRIIIb have been solved (pdb accession code 1E4K) (Sondermann *et al.*, 2000, Nature 406:267-273.) (pdb accession codes 1IIS and 1IIX) (Radaev *et al.*, 2001, J Biol Chem 276:16469-16477.)

The different IgG subclasses have different affinities for the FcγRs, with IgG1 and IgG3 typically binding substantially better to the receptors than IgG2 and IgG4 (Jefferis *et al.*, 2002, Immunol Lett 82:57-65). All FcγRs bind the same region on IgG Fc, yet with different affinities: the high affinity binder FcγRI has a K_d for IgG1 of 10^{-8} M^{-1}, whereas the low affinity receptors FcγRII and FcγRIII generally bind at 10^{-6} and 10^{-5} respectively. The extracellular domains of FcγRIIIa and FcγRIIIb are 96% identical; however, FcγRIIIb does not have an intracellular signaling domain. Furthermore, whereas FcγRI, FcγRIIa/c, and FcγRIIIa are positive regulators of immune complex-triggered activation, characterized by having an intracellular domain that has an immunoreceptor tyrosine-based activation motif (ITAM), FcγRIIb has an immunoreceptor tyrosine-based inhibition motif (ITIM) and is therefore inhibitory. Thus the former are

referred to as activation receptors, and FcγRIIb is referred to as an inhibitory receptor. The receptors also differ in expression pattern and levels on different immune cells. Yet another level of complexity is the existence of a number of FcγR polymorphisms in the human proteome. A particularly relevant polymorphism with clinical significance is V158/F158 FcγRIIIa. Human IgG1 binds with greater affinity to the V158 allotype than to the F158 allotype. This difference in affinity, and presumably its effect on ADCC and/or ADCP, has been shown to be a significant determinant of the efficacy of the anti-CD20 antibody rituximab (Rituxan®, a registered trademark of IDEC Pharmaceuticals Corporation). Subjects with the V158 allotype respond favorably to rituximab treatment; however, subjects with the lower affinity F158 allotype respond poorly (Cartron *et al.*, 2002, Blood 99:754-758). Approximately 10-20% of humans are V158/V158 homozygous, 45% are V158/F158 heterozygous, and 35-45% of humans are F158/F158 homozygous (Lehrnbecher *et al.*, 1999, Blood 94:4220-4232; Cartron *et al.*, 2002, Blood 99:754-758). Thus 80-90% of humans are poor responders, that is, they have at least one allele of the F158 FcγRIIIa.

The Fc region is also involved in activation of the complement cascade. In the classical complement pathway, C1 binds with its C1q subunits to Fc fragments of IgG or IgM, which has formed a complex with antigen(s). In certain embodiments of the invention, modifications to the Fc region comprise modifications that alter (either enhance or decrease) the ability of a Fzd-specific antibody as described herein to activate the complement system (see *e.g.*, U.S. Patent 7,740,847). To assess complement activation, a complement-dependent cytotoxicity (CDC) assay may be performed (See, *e.g.*, Gazzano-Santoro *et al.*, J. Immunol. Methods, 202:163 (1996)).

Thus in certain embodiments, the present invention provides anti-Fzd antibodies having a modified Fc region with altered functional properties, such as reduced or enhanced CDC, ADCC, or ADCP activity, or enhanced binding affinity for a specific FcγR or increased serum half-life. Other modified Fc regions contemplated herein are described, for example, in issued U.S. Patents 7,317,091; 7,657,380; 7,662,925; 6,538,124; 6,528,624; 7,297,775; 7,364,731;

Published U.S. Applications US2009092599; US20080131435; US20080138344; and published International Applications WO2006/105338; WO2004/063351; WO2006/088494; WO2007/024249.

In certain embodiments, Wnt surrogate molecules comprise antibody variable domains with the desired binding specificities fused to immunoglobulin constant domain sequences. In certain embodiments, the fusion is with an Ig heavy chain constant domain, comprising at least part of the hinge, C_H2, and C_H3 regions. In particular embodiments, the first heavy-chain constant region (C_H1) containing the site necessary for light chain bonding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host cell. This provides for greater flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yield of the desired bispecific antibody. It is, however, possible to insert the coding sequences for two or all three polypeptide chains into a single expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios have no significant effect on the yield of the desired chain combination.

Wnt surrogate molecules disclosed herein may also be modified to include an epitope tag or label, *e.g.*, for use in purification or diagnostic applications. There are many linking groups known in the art for making antibody conjugates, including, for example, those disclosed in U.S. Pat. No. 5,208,020 or EP Patent 0 425 235 B1, and Chari *et al.*, Cancer Research 52: 127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

In certain embodiments, anti-LRP5/6 antibodies and antigen-binding fragments thereof and/or anti-Fzd antibodies and antigen-binding

fragments thereof present within a Wnt surrogate molecule are monoclonal. In certain embodiments, they are humanized.

The present invention further provides in certain embodiments an isolated nucleic acid encoding a polypeptide present in a Wnt surrogate molecule disclosed herein. Nucleic acids include DNA and RNA. These and related embodiments may include polynucleotides encoding antibody fragments that bind one or more Fzd receptors and/or LRP5 or LRP6 as described herein. The term "isolated polynucleotide" as used herein shall mean a polynucleotide of genomic, cDNA, or synthetic origin, or some combination thereof, which by virtue of its origin, the isolated polynucleotide: (1) is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature; (2) is linked to a polynucleotide to which it is not linked in nature, or (3) does not occur in nature as part of a larger sequence. An isolated polynucleotide may include naturally occurring and/or artificial sequences.

The term "operably linked" means that the components to which the term is applied are in a relationship that allows them to carry out their inherent functions under suitable conditions. For example, a transcription control sequence "operably linked" to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

The term "control sequence" as used herein refers to polynucleotide sequences that can affect expression, processing or intracellular localization of coding sequences to which they are ligated or operably linked. The nature of such control sequences may depend upon the host organism. In particular embodiments, transcription control sequences for prokaryotes may include a promoter, ribosomal binding site, and transcription termination sequence. In other particular embodiments, transcription control sequences for eukaryotes may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences, transcription termination sequences and polyadenylation sequences. In certain embodiments,

"control sequences" can include leader sequences and/or fusion partner sequences.

The term "polynucleotide" as referred to herein means single-stranded or double-stranded nucleic acid polymers. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Said modifications include base modifications such as bromouridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single and double stranded forms of DNA.

The term "naturally occurring nucleotides" includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" includes nucleotides with modified or substituted sugar groups and the like. The term "oligonucleotide linkages" includes oligonucleotide linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate, phosphoroamidate, and the like. See, *e.g.*, LaPlanche *et al.*, 1986, Nucl. Acids Res., 14:9081; Stec *et al.*, 1984, J. Am. Chem. Soc., 106:6077; Stein *et al.*, 1988, Nucl. Acids Res., 16:3209; Zon *et al.*, 1991, Anti-Cancer Drug Design, 6:539; Zon *et al.*, 1991, OLIGONUCLEOTIDES AND ANALOGUES: A PRACTICAL APPROACH, pp. 87-108 (F. Eckstein, Ed.), Oxford University Press, Oxford England; Stec et al., U.S. Pat. No. 5,151,510; Uhlmann and Peyman, 1990, Chemical Reviews, 90:543, the disclosures of which are hereby incorporated by reference for any purpose. An oligonucleotide can include a detectable label to enable detection of the oligonucleotide or hybridization thereof.

The term "vector" is used to refer to any molecule (*e.g.*, nucleic acid, plasmid, or virus) used to transfer coding information to a host cell. The term "expression vector" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control

expression of inserted heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present.

As will be understood by those skilled in the art, polynucleotides may include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the skilled person.

As will be also recognized by the skilled artisan, polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide according to the present disclosure, and a polynucleotide may, but need not, be linked to other molecules and/or support materials. Polynucleotides may comprise a native sequence or may comprise a sequence that encodes a variant or derivative of such a sequence.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encodes an antibody as described herein. Some of these polynucleotides bear minimal sequence identity to the nucleotide sequence of the native or original polynucleotide sequence encoding a polypeptide within a Wnt surrogate molecule. Nonetheless, polynucleotides that vary due to differences in codon usage are expressly contemplated by the present disclosure. In certain embodiments, sequences that have been codon-optimized for mammalian expression are specifically contemplated.

Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, may be employed for the preparation of variants and/or derivatives of the polypeptides described herein.

By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provide a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments, the inventors contemplate the mutagenesis of the polynucleotide sequences that encode a polypeptide present in a Wnt surrogate molecule, to alter one or more properties of the encoded polypeptide, such as the binding affinity, or the function of a particular Fc region, or the affinity of the Fc region for a particular FcγR. The techniques of site-specific mutagenesis are well-known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phages are readily commercially available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site

directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

In many embodiments, one or more nucleic acids encoding a polypeptide of a Wnt surrogate molecule are introduced directly into a host cell, and the cell incubated under conditions sufficient to induce expression of the encoded polypeptides. The Wnt surrogate polypeptides of this disclosure may be prepared using standard techniques well known to those of skill in the art in combination with the polypeptide and nucleic acid sequences provided herein. The polypeptide sequences may be used to determine appropriate nucleic acid sequences encoding the particular polypeptide disclosed thereby. The nucleic acid sequence may be optimized to reflect particular codon "preferences" for various expression systems according to standard methods well known to those of skill in the art.

According to certain related embodiments there is provided a recombinant host cell which comprises one or more constructs as described herein, e.g., a vector comprising a nucleic acid encoding a Wnt surrogate molecule or polypeptide thereof; and a method of production of the encoded product, which method comprises expression from encoding nucleic acid therefor. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression, an antibody or antigen-binding fragment

thereof, may be isolated and/or purified using any suitable technique, and then used as desired.

Polypeptides, and encoding nucleic acid molecules and vectors, may be isolated and/or purified, *e.g.* from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes of origin other than the sequence encoding a polypeptide with the desired function. Nucleic acid may comprise DNA or RNA and may be wholly or partially synthetic. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is *E. coli*.

The expression of polypeptides, e.g., antibodies and antigen-binding fragments thereof, in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Pluckthun, A. Bio/Technology 9: 545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of antibodies or antigen-binding fragments thereof, see recent reviews, for example Ref, M. E. (1993) Curr. Opinion Biotech. 4: 573-576; Trill J. J. *et al.* (1995) Curr. Opinion Biotech 6: 553-560.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral *e.g.* phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook *et al.*, 1989, Cold Spring

Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Second Edition, Ausubel *et al.* eds., John Wiley & Sons, 1992, or subsequent updates thereto.

The term "host cell" is used to refer to a cell into which has been introduced, or which is capable of having introduced into it, a nucleic acid sequence encoding one or more of the herein described polypeptides, and which further expresses or is capable of expressing a selected gene of interest, such as a gene encoding any herein described polypeptide. The term includes the progeny of the parent cell, whether or not the progeny are identical in morphology or in genetic make-up to the original parent, so long as the selected gene is present. Accordingly there is also contemplated a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, *e.g.* vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. The introduction may be followed by causing or allowing expression from the nucleic acid, *e.g.* by culturing host cells under conditions for expression of the gene. In one embodiment, the nucleic acid is integrated into the genome (*e.g.* chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance-with standard techniques.

The present invention also provides, in certain embodiments, a method which comprises using a construct as stated above in an expression system in order to express a particular polypeptide such as a Wnyt mimetic molecule as described herein. The term "transduction" is used to refer to the transfer of genes from one bacterium to another, usually by a phage.

"Transduction" also refers to the acquisition and transfer of eukaryotic cellular sequences by retroviruses. The term "transfection" is used to refer to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. See, *e.g.*, Graham *et al.*, 1973, Virology 52:456; Sambrook *et al.*, 2001, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Spring Harbor Laboratories; Davis *et al.*, 1986, BASIC METHODS IN MOLECULAR BIOLOGY, Elsevier; and Chu *et al.*, 1981, Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transfection or transduction, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell. The term "naturally occurring" or "native" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and the like, refers to materials which are found in nature and are not manipulated by a human. Similarly, "non-naturally occurring" or "non-native" as used herein refers to a material that is not found in nature or that has been structurally modified or synthesized by a human.

The terms "polypeptide" "protein" and "peptide" and "glycoprotein" are used interchangeably and mean a polymer of amino acids not limited to any particular length. The term does not exclude modifications such as myristylation, sulfation, glycosylation, phosphorylation and addition or deletion of signal sequences. The terms "polypeptide" or "protein" means one or more chains of amino acids, wherein each chain comprises amino acids covalently linked by peptide bonds, and wherein said polypeptide or protein can comprise a plurality

of chains non-covalently and/or covalently linked together by peptide bonds, having the sequence of native proteins, that is, proteins produced by naturally-occurring and specifically non-recombinant cells, or genetically-engineered or recombinant cells, and comprise molecules having the amino acid sequence of the native protein, or molecules having deletions from, additions to, and/or substitutions of one or more amino acids of the native sequence. The terms "polypeptide" and "protein" specifically encompass Wnt surrogate molecules, Fzd binding regions thereof, LRP5/6 binding regions thereof, antibodies and antigen-binding fragments thereof that bind to a Fzd receptor or a LRP5 or LRP6 receptor disclosed herein, or sequences that have deletions from, additions to, and/or substitutions of one or more amino acid of any of these polyppetides. Thus, a "polypeptide" or a "protein" can comprise one (termed "a monomer") or a plurality (termed "a multimer") of amino acid chains.

The term "isolated protein," "isolated Wnt surrogate molecule or "isolated antibody" referred to herein means that a subject protein, Wnt surrogate molecule, or antibody: (1) is free of at least some other proteins with which it would typically be found in nature; (2) is essentially free of other proteins from the same source, *e.g.*, from the same species, (3) is expressed by a cell from a different species; (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature; (5) is not associated (by covalent or noncovalent interaction) with portions of a protein with which the "isolated protein" is associated in nature; (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature; or (7) does not occur in nature. Such an isolated protein can be encoded by genomic DNA, cDNA, mRNA or other RNA, or may be of synthetic origin, or any combination thereof. In certain embodiments, an isolated protein may comprise naturally-occurring and/or artificial polypeptide sequences. In certain embodiments, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural environment that

would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

Amino acid sequence modification(s) of any of the polypeptides (e.g., Wnt surrogate molecules or Fzd binding regions or LRP5/6 binding regions thereof) described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the Wnt surrogate molecule. For example, amino acid sequence variants of a Wnt surrogate molecule may be prepared by introducing appropriate nucleotide changes into a polynucleotide that encodes the antibody, or a chain thereof, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution may be made to arrive at the final Wnt surrogate molecule, provided that the final construct possesses the desired characteristics (*e.g.*, high affinity binding to one or more Fzd and/or LRP5/6 receptor). The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites. Any of the variations and modifications described above for polypeptides of the present invention may be included in antibodies of the present invention.

The present disclosure provides variants of any of the polypeptides (e.g., Wnt surrogate molecules or Fzd binding regions or LRP5/6 binding regions thereof, or antibodies or antigen-binding fragments thereof) disclosed herein. In certain embodiments, a variant has at least 90%, at least 95%, at least 98%, or at least 99% identity to a polypeptide disclosed herein. In certain embodiments, such variant polypeptides bind to one or more Fzd receptor, and/or to one or more LRP5/6 receptor, at least about 50%, at least about 70%, and in certain embodiments, at least about 90% as well as a Wnt surrogate molecule specifically set forth herein. In further embodiments, such variant Wnt surrogate molecules bind to one or more Fzd receptor, and/or to one or more LRP5/6 receptor, with greater affinity than the Wnt surrogate molecules set forth herein,

for example, that bind quantitatively at least about 105%, 106%, 107%, 108%, 109%, or 110% as well as an antibody sequence specifically set forth herein.

In particular embodiments, the Wnt surrogate molecule or a binding region thereof, e.g., a Fab, scFv, or VHH or sdAb may comprise: a) a heavy chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the heavy chain CDR1 region of a selected antibody described herein; ii. a CDR2 region that is identical in amino acid sequence to the heavy chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the heavy chain CDR3 region of the selected antibody; and/or b) a light chain variable domain comprising: i. a CDR1 region that is identical in amino acid sequence to the light chain CDR1 region of the selected antibody; ii. a CDR2 region that is identical in amino acid sequence to the light chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the light chain CDR3 region of the selected antibody; wherein the antibody specifically binds a selected target (e.g., one or more Fzd receptors or LRP5 or LRP6 receptors). In a further embodiment, the antibody, or antigen-binding fragment thereof, is a variant antibody or antigen-binding fragment thereof wherein the variant comprises a heavy and light chain identical to the selected antibody except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions in the CDR regions of the VH and VL regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more amino acid substitutions in the CDR regions of the selected antibody. Substitutions may be in CDRs either in the VH and/or the VL regions. (See *e.g.*, Muller, 1998, Structure 6:1153-1167).

In particular embodiments, the Wnt surrogate molecule or a binding region thereof, e.g., a Fab, scFv, or VHH or sdAb may have: a) a heavy chain variable region having an amino acid sequence that is at least 80% identical, at least 95% identical, at least 90%, at least 95% or at least 98% or 99% identical, to the heavy chain variable region of an antibody or antigen-binding fragments thereof described herein; and/or b) a light chain variable region having an amino acid sequence that is at least 80% identical, at least 85%, at least 90%, at least

95% or at least 98% or 99% identical, to the light chain variable region of an antibody or antigen-binding fragments thereof described herein. The amino acid sequence of illustrative antigen-binding fragments thereof are set forth in SEQ ID NOs:1-128.

A polypeptide has a certain percent "sequence identity" to another polypeptide, meaning that, when aligned, that percentage of amino acids are the same when comparing the two sequences. Sequence similarity can be determined in a number of different manners. To determine sequence identity, sequences can be aligned using the methods and computer programs, including BLAST, available over the world wide web at ncbi.nlm.nih.gov/BLAST/. Another alignment algorithm is FASTA, available in the Genetics Computing Group (GCG) package, from Madison, Wis., USA, a wholly owned subsidiary of Oxford Molecular Group, Inc. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle, Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, Calif., USA. Of particular interest are alignment programs that permit gaps in the sequence. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See Meth. Mol. Biol. 70: 173-187 (1997). Also, the GAP program using the Needleman and Wunsch alignment method can be utilized to align sequences. See J. Mol. Biol. 48: 443-453 (1970)

Of interest is the BestFit program using the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2: 482-489 (1981) to determine sequence identity. The gap generation penalty will generally range from 1 to 5, usually 2 to 4 and in many embodiments will be 3. The gap extension penalty will generally range from about 0.01 to 0.20 and in many instances will be 0.10. The program has default parameters determined by the sequences inputted to be compared. Preferably, the sequence identity is determined using the default parameters determined by the program. This program is available also from Genetics Computing Group (GCG) package, from Madison, Wis., USA.

Another program of interest is the FastDB algorithm. FastDB is described in Current Methods in Sequence Comparison and Analysis, Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp. 127-149, 1988, Alan R. Liss, Inc. Percent sequence identity is calculated by FastDB based upon the following parameters:
Mismatch Penalty: 1.00; Gap Penalty: 1.00; Gap Size Penalty: 0.33; and Joining Penalty: 30.0.

In particular embodiments, the Wnt surrogate molecule or a binding region thereof, e.g., a Fab, scFv, or VHH or sdAb may comprise: a) a heavy chain variable region comprising: i. a CDR1 region that is identical in amino acid sequence to the heavy chain CDR1 region of a selected antibody described herein; ii. a CDR2 region that is identical in amino acid sequence to the heavy chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the heavy chain CDR3 region of the selected antibody; and b) a light chain variable domain comprising: i. a CDR1 region that is identical in amino acid sequence to the light chain CDR1 region of the selected antibody; ii. a CDR2 region that is identical in amino acid sequence to the light chain CDR2 region of the selected antibody; and iii. a CDR3 region that is identical in amino acid sequence to the light chain CDR3 region of the selected antibody; wherein the antibody specifically binds a selected target (e.g., a Fzd receptor, such as Fzd1). In a further embodiment, the antibody, or antigen-binding fragment thereof, is a variant antibody wherein the variant comprises a heavy and light chain identical to the selected antibody except for up to 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions in the CDR regions of the VH and VL regions. In this regard, there may be 1, 2, 3, 4, 5, 6, 7, 8, or in certain embodiments, 9, 10, 11, 12, 13, 14, 15 more amino acid substitutions in the CDR regions of the selected antibody. Substitutions may be in CDRs either in the VH and/or the VL regions. (See *e.g.*, Muller, 1998, Structure 6:1153-1167).

Determination of the three-dimensional structures of representative polypeptides (*e.g.*, variant Fzd binding regions or LRP5/6 binding regions of Wnt surrogate molecules as provided herein) may be made through routine

methodologies such that substitution, addition, deletion or insertion of one or more amino acids with selected natural or non-natural amino acids can be virtually modeled for purposes of determining whether a so derived structural variant retains the space-filling properties of presently disclosed species. *See,* for instance, Donate et al., 1994 *Prot. Sci.* 3:2378; Bradley et al., *Science* 309: 1868-1871 (2005); Schueler-Furman et al., *Science* 310:638 (2005); Dietz et al., *Proc. Nat. Acad. Sci. USA* 103:1244 (2006); Dodson et al., *Nature* 450:176 (2007); Qian et al., *Nature* 450:259 (2007); Raman *et al.* Science 327:1014-1018 (2010). Some additional non-limiting examples of computer algorithms that may be used for these and related embodiments, such as for rational design of binding regions include VMD which is a molecular visualization program for displaying, animating, and analyzing large biomolecular systems using 3-D graphics and built-in scripting (see the website for the Theoretical and Computational Biophysics Group, University of Illinois at Urbana-Champagne, at ks.uiuc.edu/Research/vmd/. Many other computer programs are known in the art and available to the skilled person and which allow for determining atomic dimensions from space-filling models (van der Waals radii) of energy-minimized conformations; GRID, which seeks to determine regions of high affinity for different chemical groups, thereby enhancing binding, Monte Carlo searches, which calculate mathematical alignment, and CHARMM (Brooks et al. (1983) *J. Comput. Chem.* 4:187-217) and AMBER (Weiner et al (1981) *J. Comput. Chem*. 106: 765), which assess force field calculations, and analysis (see also, Eisenfield et al. (1991) *Am. J. Physiol.* 261:C376-386; Lybrand (1991) *J. Pharm. Belg.* 46:49-54; Froimowitz (1990) *Biotechniques* 8:640-644; Burbam et al. (1990) *Proteins* 7:99-111; Pedersen (1985) *Environ. Health Perspect.* 61:185-190; and Kini et al. (1991) *J. Biomol. Struct. Dyn.* 9:475-488). A variety of appropriate computational computer programs are also commercially available, such as from Schrödinger (Munich, Germany).

Compositions

Pharmaceutical compositions comprising a Wnt surrogate molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or

excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises one or more Wnt polypeptides or Norrin polypeptides.

In further embodiments, pharmaceutical compositions comprising a polynucleotide comprising a nucleic acid sequence encoding a Wnt surrogate molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises one or more polynucleotides comprising a nucleic acid sequence encoding a Wnt polypeptide or Norrin polypeptide. In certain embodiments, the polynucleotides are DNA or mRNA, e.g., a modified mRNA. In particular embodiments, the polynucleotides are modified mRNAs further comprising a 5' cap sequence and/or a 3' tailing sequence, e.g., a polyA tail. In other embodiments, the polynucleotides are expression cassettes comprising a promoter operatively linked to the coding sequences. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same polynucleotide.

In further embodiments, pharmaceutical compositions comprising an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a Wnt surrogate molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In particular embodiments, the pharmaceutical composition further comprises an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a Wnt polypeptide or Norrin polypeptide. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same polynucleotide, e.g., expression cassette.

The present invention further contemplates a pharmaceutical composition comprising a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid

encoding a Wnt surrogate molecule and one or more pharmaceutically acceptable diluent, carrier, or excipient. In particular embodiments, the pharmaceutical composition further comprises a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid sequence encoding a Wnt polypeptide or a Norrin polypeptide. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same polynucleotide, e.g., expression cassette and/or in the same cell. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated. In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell.

The present disclosure contemplates pharmaceutical compositions comprising a first molecule for delivery of a Wnt surrogate molecule as a first active agent and a second molecule for delivery of a Wnt polypeptide or Norrin polypeptide. The first and second molecule may be the same type of molecule or different types of molecules. For example, in certain embodiments, the first and second molecule may each be independently selected from the following types of molecules: polypeptides, small organic molecules, nucleic acids encoding the first or second active agent (optionally DNA or mRNA, optionally modified RNA), vectors comprising a nucleic acid sequence encoding the first or second active agent (optionally expression vectors or viral vectors), and cells comprising a nucleic acid sequence encoding the first or second active agent (optionally an expression cassette).

The subject molecules, alone or in combination, can be combined with pharmaceutically-acceptable carriers, diluents, excipients and reagents useful in preparing a formulation that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for mammalian, e.g., human or primate, use. Such excipients can be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. Examples of such carriers, diluents and excipients include, but are not limited to, water, saline, Ringer's solutions, dextrose solution,

and 5% human serum albumin. Supplementary active compounds can also be incorporated into the formulations. Solutions or suspensions used for the formulations can include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates; detergents such as Tween 20 to prevent aggregation; and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. In particular embodiments, the pharmaceutical compositions are sterile.

Pharmaceutical compositions may further include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, or phosphate buffered saline (PBS). In some cases, the composition is sterile and should be fluid such that it can be drawn into a syringe or delivered to a subject from a syringe. In certain embodiments, it is stable under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be, e.g., a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the internal compositions can be brought

about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile solutions can be prepared by incorporating the anti-Fzd antibody or antigen-binding fragment thereof (or encoding polynucleotide or cell comprising the same) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In one embodiment, the pharmaceutical compositions are prepared with carriers that will protect the antibody or antigen-binding fragment thereof against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

It may be advantageous to formulate the pharmaceutical compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active antibody or antigen-binding fragment thereof calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms are dictated by and directly dependent on the unique characteristics of the antibody

or antigen-binding fragment thereof and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active antibody or antigen-binding fragment thereof for the treatment of individuals.

The pharmaceutical compositions can be included in a container, pack, or dispenser, e.g. syringe, e.g. a prefilled syringe, together with instructions for administration.

The pharmaceutical compositions of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal comprising a human, is capable of providing (directly or indirectly) the biologically active antibody or antigen-binding fragment thereof.

The present invention includes pharmaceutically acceptable salts of a Wnt surrogate molecule described herein. The term “pharmaceutically acceptable salt” refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. A variety of pharmaceutically acceptable salts are known in the art and described, e.g., in “Remington’s Pharmaceutical Sciences”, 17th edition, Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, USA, 1985 (and more recent editions thereof), in the “Encyclopaedia of Pharmaceutical Technology”, 3rd edition, James Swarbrick (Ed.), Informa Healthcare USA (Inc.), NY, USA, 2007, and in J. Pharm. Sci. 66: 2 (1977). Also, for a review on suitable salts, see “Handbook of Pharmaceutical Salts: Properties, Selection, and Use” by Stahl and Wermuth (Wiley-VCH, 2002).

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Metals used as cations comprise sodium, potassium, magnesium, calcium, and the like. Amines comprise N-N’-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., “Pharmaceutical Salts,” J. Pharma Sci., 1977, 66, 119). The base addition salts of said acidic

compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

In some embodiments, the pharmaceutical composition provided herein comprise a therapeutically effective amount of a Wnt surrogate molecule or pharmaceutically acceptable salt thereof in admixture with a pharmaceutically acceptable carrier, diluent and/or excipient, for example saline, phosphate buffered saline, phosphate and amino acids, polymers, polyols, sugar, buffers, preservatives and other proteins. Exemplary amino acids, polymers and sugars and the like are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds, polyoxyethylene sorbitan fatty acid esters, sucrose, fructose, dextrose, maltose, glucose, mannitol, dextran, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, bovine or human serum albumin, citrate, acetate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine, polyvinylpyrrolidone, polyethylene and glycol. Preferably, this formulation is stable for at least six months at 4º C.

In some embodiments, the pharmaceutical composition provided herein comprises a buffer, such as phosphate buffered saline (PBS) or sodium phosphate/sodium sulfate, tris buffer, glycine buffer, sterile water and other buffers known to the ordinarily skilled artisan such as those described by Good et al. (1966) Biochemistry 5:467. The pH of the buffer may be in the range of 6.5 to 7.75, preferably 7 to 7.5, and most preferably 7.2 to 7.4.

Methods of Use

The present disclosure also provides methods for using the Wnt surrogate molecules disclosed herein, e.g., to modulate a Wnt signaling pathway, e.g., to increase Wnt signaling, and the administration of a Wnt surrogate molecule disclosed herein in a variety of therapeutic settings. Provided herein

are methods of treatment using a Wnt surrogate molecule. In one embodiment, a Wnt surrogate molecule is provided to a subject having a disease involving inappropriate or deregulated Wnt signaling, e.g., reduced Wnt signaling.

Increasing Wnt Pathway Signaling and Related Therapeutic Methods

In certain embodiments, a Wnt surrogate molecule may be used to increase Wnt signaling in a tissue or cell. Thus, in some aspects, the present invention provides a method for increasing Wnt signaling or enhancing Wnt signaling in a tissue or cell, comprising contacting the tissue or cell with an effective amount of a Wnt surrogate molecule or pharmaceutically acceptable salt thereof disclosed herein, wherein the a Wnt surrogate molecule is a Wnt signaling pathway agonist. In some embodiments, contacting occurs in vitro, ex vivo, or in vivo. In particular embodiments, the cell is a cultured cell, and the contacting occurs in vitro. In certain embodiments, the method comprises further contacting the tissue or cell with one or more Wnt polypeptides or Norrin polypeptides.

In related aspects, the present invention provides a method for increasing Wnt signaling in a tissue or cell, comprising contacting the tissue or cell with an effective amount of a polynucleotide comprising a Wnt surrogate molecule disclosed herein. In certain embodiments, the target tissue or cell is also contacted with a polynucleotide comprising a nucleic acid sequence that encodes a Wnt polypeptide or a Norrin polypeptide. In certain embodiments, the polynucleotides are DNA or mRNA, e.g., a modified mRNA. In particular embodiments, the polynucleotides are modified mRNAs further comprising a 5' cap sequence and/or a 3' tailing sequence, e.g., a polyA tail. In other embodiments, the polynucleotides are expression cassettes comprising a promoter operatively linked to the coding sequences. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same polynucleotide.

In related aspects, the present invention provides a method for increasing Wnt signaling in a tissue or cell, comprising contacting the tissue or cell

with an effective amount of a vector comprising a nucleic acid sequence encoding a Wnt surrogate molecule. In certain embodiments, the tissue or cell is also contacted with a vector comprising a nucleic acid sequence that encodes a Wnt polypeptide or a Norrin polypeptide. In certain embodiments, the vector is an expression vector, and may comprise a promoter operatively linked to the nucleic acid sequence. In particular embodiments, the vector is a viral vector. In certain embodiments, the nucleic acid sequence encoding a Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same vector, e.g., in the same expression cassette.

In related aspects, the present invention provides a method for increasing Wnt signaling in a tissue, comprising contacting the tissue with an effective amount of a cell comprising a nucleic acid sequence encoding a Wnt surrogate molecule of the present invention. In certain embodiments, the tissue is also contacted with a cell comprising a nucleic acid sequence that encodes a Wnt polypeptide or Norrin polypeptide. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same cell. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated. In certain embodiments, the cell was transduced with a vector comprising an expression cassette encoding the Wnt surrogate molecule or the Wnt polypeptide or Norrin polypeptide. In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell.

Wnt surrogate molecules disclosed herein may be used in to treat a disease, disorder or condition, for example, by increasing Wnt signaling in a targeted cell, tissue or organ. Thus, in some aspects, the present invention provides a method for treating a disease or condition in a subject in need thereof, e.g., a disease or disorder associated with reduced Wnt signaling, or for which increased Wnt signaling would provide a therapeutic benefit, comprising contacting the subject with an effective amount of a composition of the present disclosure. In particular embodiments, the composition is a pharmaceutical

composition comprising any of: a Wnt surrogate molecule; a polynucleotide comprising a nucleic acid sequence encoding a Wnt surrogate molecule, e.g., a DNA or mRNA, optionally a modified mRNA; a vector comprising a nucleic acid sequence encoding a Wnt surrogate molecule, e.g., an expression vector or viral vector; or a cell comprising a nucleic acid sequence encoding a Wnt surrogate molecule, e.g., a cell transduced with an expression vector or viral vector encoding a Wnt surrogate molecule. In particular embodiments, the disease or condition is a pathological disease or disorder, or an injury, e.g., an injury resulting from a wound. In certain embodiments, the wound may be the result of another therapeutic treatment. In certain embodiments, the disease or condition comprises impaired tissue repair, healing or regeneration, or would benefit from increased tissue repair, healing or regeneration. In some embodiments, contacting occurs in vivo, i.e., the subject composition is administered to a subject.

In certain embodiments, the method comprises further contacting the subject with a pharmaceutical composition comprising one or more Wnt polypeptides or Norrin polypeptides. The present disclosure contemplates contacting a subject with a first molecule for delivery of a Wnt surrogate molecule as a first active agent and a second molecule for delivery of a Wnt polypeptide or Norrin polypeptide. The first and second molecule may be the same type of molecule or different types of molecules. For example, in certain embodiments, the first and second molecule may each be independently selected from the following types of molecules: polypeptides, small organic molecules, nucleic acids encoding the first or second active agent (optionally DNA or mRNA, optionally modified RNA), vectors comprising a nucleic acid sequence encoding the first or second active agent (optionally expression vectors or viral vectors), and cells comprising a nucleic acid sequence encoding the first or second active agent (optionally an expression cassette).

In related aspects, the present invention provides a method for treating a disease or condition, e.g., a disease or disorder associated with reduced Wnt signaling, or for which increased Wnt signaling would provide a

therapeutic benefit, comprising contacting a subject in need thereof with a pharmaceutical composition comprising an effective amount of a polynucleotide comprising a nucleic acid sequence encoding a Wnt surrogate molecule disclosed herein. In certain embodiments, the subject is also contacted with a pharmaceutical composition comprising an effective amount of a polynucleotide comprising a nucleic acid sequence that encodes a Wnt polypeptide or a Norrin polypeptide. In certain embodiments, the polynucleotides are DNA or mRNA, e.g., a modified mRNA. In particular embodiments, the polynucleotides are modified mRNAs further comprising a 5' cap sequence and/or a 3' tailing sequence, e.g., a polyA tail. In other embodiments, the polynucleotides are expression cassettes comprising a promoter operatively linked to the coding sequences. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same polynucleotide.

In related aspects, the present invention provides a method for treating a disease or condition, e.g., a disease or disorder associated with reduced Wnt signaling, or for which increased Wnt signaling would provide a therapeutic benefit, comprising contacting a subject in need thereof with a pharmaceutical composition comprising an effective amount of a vector comprising a nucleic acid sequence encoding a Wnt surrogate molecule. In certain embodiments, the subject is also contacted with a pharmaceutical composition comprising an effective amount of a vector comprising a nucleic acid sequence that encodes a Wnt polypeptide or a Norrin polypeptide. In certain embodiments, the vector is an expression vector, and may comprise a promoter operatively linked to the nucleic acid sequence. In particular embodiments, the vector is a viral vector. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same vector, e.g., in the same expression cassette.

In related aspects, the present invention provides a method for treating a disease or condition, e.g., a disease or disorder associated with

reduced Wnt signaling, or for which increased Wnt signaling would provide a therapeutic benefit, comprising contacting a subject in need thereof with a pharmaceutical composition comprising an effective amount of a cell comprising a nucleic acid sequence encoding a Wnt surrogate molecule. In certain embodiments, the subject is also contacted with a cell comprising a nucleic acid sequence that encodes a Wnt polypeptide or a Norrin polypeptide. In certain embodiments, the nucleic acid sequence encoding the Wnt surrogate molecule and the nucleic acid sequence encoding the Wnt polypeptide or Norrin polypeptide are present in the same cell. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated. In certain embodiments, the cell was transduced with a vector comprising an expression cassette encoding the Wnt surrogate molecule or the Wnt polypeptide or Norrin polypeptide. In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell.

Wnt signaling plays key roles in the developmental process and maintenance of stem cells. Reactivation of Wnt signals is associated with regeneration and repair of most tissues after injuries and diseases. Wnt surrogate molecule molecules are expected to provide benefit of healing and tissue repair in response to injuries and diseases. Causes of tissue damage and loss include but are not limited to aging, degeneration, hereditary conditions, infection and inflammation, traumatic injuries, toxins/metabolic-induced toxicities, or other pathological conditions. Wnt signals and enhancers of Wnt signals have been shown to activate adult, tissue-resident stem cells. In some embodiments, the compounds of the invention are administered for use in treating diseased or damaged tissue, for use in tissue regeneration and for use in cell growth and proliferation, and/or for use in tissue engineering.

Human diseases associated with mutations of the Wnt pathway provide strong evidence for enhancement of Wnt signals in the treatment and prevention of diseases. Preclinical in vivo and in vitro studies provide additional evidence of involvement of Wnt signals in many disease conditions and further support utilization of a Wnt surrogate molecule in various human diseases. For

example, compositions of the present invention may be used to promote or increase bone growth or regeneration, bone grafting, healing of bone fractures, treatment of osteoporosis and osteoporotic fractures, spinal fusion, spinal cord injuries, including vertebral compression fractures, pre-operative spinal surgery optimization, osseointegration of orthopedic devices, tendon-bone integration, tooth growth and regeneration, dental implantation, periodontal diseases, maxillofacial reconstruction, and osteonecrosis of the jaw. They may also be used in the treatment of alopecia; enhancing regeneration of sensory organs, e.g. treatment of hearing loss, including regeneration of inner and outer auditory hair cells treatment of vestibular hypofunction, treatment of macular degeneration, treatment of retinopathies, including vitreoretinopathy, diabetic retinopathy, other diseases of retinal degeneration, Fuchs' dystrophy, other cornea disease, etc.; treatment of stroke, traumatic brain injury, Alzheimer's disease, multiple sclerosis, multiple dystrophy, muscle atrophy as a result of sarcopenia or cachexia, and other conditions affecting the degeneration or integrity of the blood brain barrier; . The compositions of this invention may also be used in treatment of oral mucositis, treatment of short bowel syndrome, inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), in particular CD with fistula formation, other gastrointestinal disorders; treatment of metabolic syndrome, dyslipidemia, treatment of diabetes, treatment of pancreatitis, conditions where exocrine or endocrine pancreas tissues are damaged; conditions where enhanced epidermal regeneration is desired, e.g., epidermal wound healing, treatment of diabetic foot ulcers, syndromes involving tooth, nail, or dermal hypoplasia, etc., conditions where angiogenesis is beneficial; treatment of myocardial infarction, coronary artery disease, heart failure; enhanced growth of hematopoietic cells, e.g. enhancement of hematopoietic stem cell transplants from bone marrow, mobilized peripheral blood, treatment of immunodeficiencies, graft versus host diseases, etc.; treatment of acute kidney injuries, chronic kidney diseases; treatment of lung diseases, chronic obstructive pulmonary diseases (COPD), pulmonary fibrosis, including idiopathic pulmonary fibrosis, enhanced

regeneration of lung tissues. The compositions of the present invention may also be used in enhanced regeneration of liver cells, e.g. liver regeneration, treatment of cirrhosis, enhancement of liver transplantations, treatment of acute liver failure, treatment of chronic liver diseases with hepatitis C or B virus infection or post-antiviral drug therapies, alcoholic liver diseases, including alcoholic hepatitis, non-alcoholic liver diseases with steatosis or steatohepatitis, and the like. The compositions of this invention may treat diseases and disorders including, without limitation, conditions in which regenerative cell growth is desired.

Human genetics involving loss-of-function or gain-of-function mutations in Wnt signaling components show strong evidence supporting enhancing Wnt signals for bone growth. Conditions in which enhanced bone growth is desired may include, without limitation, fractures, grafts, ingrowth around prosthetic devices, osteoporosis, osteoporotic fractures, spinal fusion, vertebral compression fractures, pre-operative optimization for spinal surgeries, osteonecrosis of the jaw, dental implantation, periodontal diseases, maxillofacial reconstruction, and the like. Wnt surrogate molecules enhance and promotes Wnt signals which are critical in promoting bone regeneration. Methods for regeneration of bone tissues benefit from administration of the compounds of the invention, which can be systemic or localized. In some embodiments, bone marrow cells are exposed to molecules of the invention, such that stem cells within that marrow become activated.

In some embodiments, bone regeneration is enhanced by contacting a responsive cell population, e.g. bone marrow, bone progenitor cells, bone stem cells, etc. with an effective dose of a Wnt surrogate molecule disclosed herein. Methods for regeneration of bone tissues benefit from administration of the Wnt surrogate molecule which can be systemic or localized. In some such embodiments, the contacting is performed in vivo. In other such embodiments, the contacting is performed ex vivo. The molecule may be localized to the site of action, e.g. by loading onto a matrix, which is optionally biodegradable, and optionally provides for a sustained release of the active

agent. Matrix carriers include, without limitation, absorbable collagen sponges, ceramics, hydrogels, polymeric microspheres, nanoparticles, bone cements, and the like.

In particular embodiments, compositions comprising one or more Wnt surrogate molecule disclosed herein (or a polynucleotide encoding a Wnt surrogate molecule, or a vector or cell comprising a polynucleotide encoding a Wnt surrogate molecule) are used to treat or prevent a bone disease or disorder, including but not limited to any of the following, or to treat or prevent an injury associated with, but not limited to, any of the following: osteoporosis, osteoporotic fractures, bone fractures including vertebral compression fractures, non-union fractures, delayed union fractures, spinal fusion, osteonecrosis, osteonecrosis of the jaw, hip, femoral head, etc., osseointegration of implants (e.g., to accelerate recovery following partial or total knee or hip replacement), osteogenesis imperfecta, bone grafts, tendon repair, maxillofacial surgery, dental implant, all other bone disorders or defects resulting from genetic diseases, degeneration, aging, drugs, or injuries. In one embodiment, Wnt surrogate molecules that bind Fzd1, Fzd 2, and Fzd 7, and also LRP5 and/or LRP6, are used to treat or prevent any bone disease or disorder. In one embodiment, Wnt surrogate molecules that bind Fzd1, Fzd 2, Fzd 5, Fzd 7 and Fzd 8, and also LRP5 and/or LRP6, are used to treat or prevent any bone disease or disorder. Other Fzd molecules that bind to additional Fzd receptors can also be used with LRP5 and/or LRP6 binders.

In particular embodiments, compositions and methods disclosed herein may be used to: increase bone mineral density, increase bone volume (e.g., tibia and/or femur bone volume), increase cortical thickness (e.g., in trabecular region or in femur mid-diaphysis), increase mineral apposition rate, increase the number of osteblasts and/or decrease the number of osteoclasts (e.g., in bone), increase bone stiffness, increase the ultimate load to fracture point, improve bone resistance to fracture, decrease bone resorption, decrease bone loss associated with osteoporosis, or increase biochemical strength of bone, in a subject. In one embodiment, Wnt surrogate molecules that bind Fzd1,

Fzd 2, and Fzd 7 are used for any of these indicated uses. In one embodiment, Wnt surrogate molecules that bind Fzd1, Fzd 2, Fzd 5, Fzd 7 and Fzd 8 are used for any of these indicated uses.

Methods disclosed herein, including methods for treating or preventing a bone disease or disorder include methods that comprise providing to a subject in need thereof both a Wnt surrogate molecule and an antiresorptive agent. In certain embodiments, the methods are used for the treatment of osteoporosis, optionally post-menopausal osteoporosis.

The disclosure also provides a method for inhibiting or reducing bone resorption in a subject in need thereof, comprising providing to the subject an effective amount of a Wnt surrogate molecule, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway. In certain embodiments, the method further comprises providing to the subject an antiresorptive agent. In certain embodiments, the subject has been diagnosed with or is at risk for osteoporosis, optionally postmenopausal osteoporosis. A variety of antiresorptive agents are known in the art and include, but are not limited to, those disclosed herein.

When a Wnt surrogate molecule is provide to the subject in combination with another therapeutic agent, such as an antiresorptive agent, the two agent may be provided in the same or different pharmaceutical compositions. They may be provided to the subject at the same time, at different times, e.g., simultaneously, consecutively, or during overlapping or non-overlapping time periods. In certain embodiments, the two agents are therapeutically active in the subject during an overlapping time period.

Compositions comprising one or more Wnt surrogate molecule disclosed herein (or a polynucleotide encoding a Wnt surrogate molecule, or a vector or cell comprising a polynucleotide encoding a Wnt surrogate molecule) can be used for the in vivo treatment of skeletal tissue deficiencies. By "skeletal tissue deficiency", it is meant a deficiency in bone or other skeletal connective tissue at any site where it is desired to restore the bone or connective tissue, no matter how the deficiency originated, e.g. whether as a result of surgical

intervention, removal of tumor, ulceration, implant, fracture, or other traumatic or degenerative conditions. The compositions of the present invention can be used as part of a regimen for restoring cartilage function to a connective tissue, for the repair of defects or lesions in cartilage tissue such as degenerative wear and arthritis, trauma to the tissue, displacement of torn meniscus, meniscectomy, a luxation of a joint by a torn ligament, malalignment of joints, bone fracture, or by hereditary disease.

A Wnt surrogate molecule may also be used for treatment of periodontal diseases. Periodontal diseases are a leading cause of tooth loss and are linked to multiple systemic conditions. In some embodiments, tooth or underlying bone regeneration is enhanced by contacting a responsive cell population. In some such embodiments, the contacting is performed in vivo. In other such embodiments, the contacting is performed ex vivo, with subsequent implantation of the activated stem or progenitor cells. The molecule may be localized to the site of action, e.g. by loading onto a matrix, which is optionally biodegradable, and optionally provides for a sustained release of the active agent. Matrix carriers include, without limitation, absorbable collagen sponges, ceramics, hydrogels, bone cements, polymeric microspheres, nanoparticles, and the like.

Studies have shown that biology of Wnt signaling and R-spondins are capable of promoting sensory hair cell regeneration in the inner ear following injuries, aging, or degeneration. Loss of sensory hair cells in the inner ear involved in hearing loss or vestibular hypofunction may also benefit from the compositions of the invention. In the inner ear, the auditory organ houses mechanosensitive hair cells required for translating sound vibration to electric impulses. The vestibular organs, comprised of the semicircular canals (SSCs), the utricle, and the saccule, also contain sensory hair cells in order to detect head position and motion. Compositions of the present invention can be used, for example, in an infusion; in a matrix or other depot system; or other topical application to the ear for enhancement of auditory regeneration.

A Wnt surrogate molecule may also be used in regeneration of retinal tissue. In the adult mammalian retina, Muller glia cells are capable of regenerating retinal cells, including photoreceptors, for example after neurotoxic injury in vivo. Wnt signaling and enhancers of Wnt signals can promote proliferation of Muller glia-derived retinal progenitors after damage or during degeneration. The compositions of the invention may also be used in the regeneration of tissues and other cell types in the eye. For examples age-related macular degeneration (AMD), other retina degenerative diseases, cornea diseases, Fuchs' dystrophy, vitreoretinopathy, hereditary diseases, etc. can benefit from the compositions of the present inventions. AMD is characterized by progressively decreased central vision and visual acuity. Fuchs' dystrophy is characterized by progressive loss of cornea endothelial cells. Wnt signal and enhancing of Wnt signal can promote regeneration of cornea endothelium, retina epithelium, etc. in the eye tissue. In other embodiments, compositions of the present invention can be used, for example, in an infusion; in a matrix or other depot system; or other topical application to the eye for retinal regeneration and treatment of macular degeneration.

Specific populations of proliferating cells for homeostatic renewal of hepatocytes have been identified through lineage tracing studies, for example Axin2-positive cells in peri-central region. Lineage tracing studies also identified additional potential liver progenitor cells, including but not limited to Lgr-positive cells. The self-renewing liver cells and other populations of potential progenitor cells, including Lgr5-positive and Axin2-positive cells, are identified to be capable of regeneration responding to Wnt signals and/or R-spondins following injuries. Numerous preclinical models of acute liver injury and failure and chronic liver diseases showed recovery and regeneration of hepatocytes benefit from enhancing Wnt signals.

In certain embodiments, compositions comprising a Wnt surrogate molecule disclosed herein (or a polynucleotide encoding a Wnt surrogate molecule, or a vector or cell comprising a polynucleotide encoding a Wnt surrogate molecule) are used to promote liver regeneration, reduce fibrosis,

and/or improve liver function. In certain embodiments, compositions and methods disclosed herein are used to: increase liver weight, increase the liver to body weight ratio, increase the number of PCNA and pH3 positive nuclei in liver, increase expression of Ki67 and/or Cyclin D1 in liver, increase liver cell proliferation and/or mitosis, decrease fibrosis following chronic liver injury, or increase hepatocyte function.

In particular embodiments, the compositions of this invention may be used in treatment of acute liver failure, acute alcoholic liver injuries, treatment of chronic liver diseases with hepatitis C or B virus infection or post-antiviral drug therapies, chronic alcoholic liver diseases, alcoholic hepatitis, non-alcoholic fatty liver diseases and non-alcoholic steatohepatitis (NASH), treatment of cirrhosis and severe chronic liver diseases of all causes, and enhanced regeneration of liver cells. Methods for regeneration of liver tissue benefit from administration of the compounds of the invention, which can be systemic or localized. These include, but are not limited to, methods of systemic administration and methods of localized administration e.g. by injection into the liver tissue, by injection into veins or blood vessels leading into the liver, by implantation of a sustained release formulation, and the like.

In particular embodiments, compositions comprising a Wnt surrogate molecule disclosed herein (or a polynucleotide encoding a Wnt surrogate molecule, or a vector or cell comprising a polynucleotide encoding a Wnt surrogate molecule) are used to treat or prevent a liver disease or disorder, including but not limited to, or to treat or prevent a liver injury or disorder resulting from any of the following: acute liver failure (all causes), chronic liver failure (all causes), cirrhosis, liver fibrosis (all causes), portal hypertension, alcoholic liver diseases including alcoholic hepatitis, nonalcoholic steatohepatisis (NASH), nonalcoholic fatty liver disease (NAFLD) (fatty liver), alcoholic hepatitis, hepatitis C virus-induced liver diseases (HCV), hepatitis B virus-induced liver diseases (HBV), other viral hepatitis (e.g., hepatitis A virus-induced liver diseases (HAV) and hepatitis D virus-induced liver diseases (HDV)), primary biliary cirrhosis, autoimmune hepatitis, livery surgery, liver injury, liver transplantation, “small for

size" syndrome in liver surgery and transplantation, congenital liver disease and disorders, any other liver disorder or detect resulting from genetic diseases, degeneration, aging, drugs, or injuries.

Wnt signals play an important role in regeneration of various epithelial tissues. Various epidermal conditions benefit from treatment with the compounds of the present invention. Mucositis occurs when there is a breakdown of the rapidly divided epithelial cells lining the gastro-intestinal tract, leaving the mucosal tissue open to ulceration and infection. The part of the epithelial lining that covers the mouth, called the oral mucosa, is one of the most sensitive parts of the body and is particularly vulnerable to chemotherapy and radiation. Oral mucositis is probably the most common, debilitating complication of cancer treatments, particularly chemotherapy and radiation. In addition, the compositions of the invention may also benefit treatment of short bowel syndrome, inflammatory bowel diseases (IBD), or other gastrointestinal disorders. Other epidermal conditions include epidermal wound healing, diabetic foot ulcers, syndromes involving tooth, nail, or dermal hypoplasia, and the like. Molecules of the present invention may be used in all these conditions, where regenerative cells are contacted with compounds of the invention. Methods for regeneration of epithelial tissues benefit from administration of the compounds of the invention, which can be systemic or localized. Contacting can be, for example, topical, including intradermal, subdermal, in a gel, lotion, cream etc. applied at targeted site, etc.

In addition to skin and gastrointestinal tract, Wnt signals and enhancement and promotion of Wnt signals also play an important role in repair and regeneration of tissues including pancreas, kidney, and lung in preclinical models. A Wnt surrogate molecule may benefit various disease conditions involving exocrine and endocrine pancreas, kidney, or lung. The Wnt surrogate molecules may be used in treatment of metabolic syndrome; treatment of diabetes, treatment of acute or chronic pancreatitis, exocrine pancreatic insufficiency, treatment of acute kidney injuries, chronic kidney diseases, treatment of lung diseases, including but not limited to chronic obstructive

pulmonary diseases (COPD), pulmonary fibrosis, in particular idiopathic pulmonary fibrosis (IPF), and other conditions that cause loss of lung epithelial tissues. Methods for regeneration of these tissues benefit from administration of the compounds of the invention, which can be systemic or localized.

Epidermal Wnt signaling, in coordination with signaling via other development factors, is critical for adult hair follicle regeneration. Hair loss is a common problem, and androgenetic alopecia, often called male pattern baldness, is the most common form of hair loss in men. In some embodiments, hair follicle regeneration is enhanced by contacting a responsive cell population with a molecule of the present invention. In some such embodiments, the contacting is performed in vivo. In other such embodiments, the contacting is performed ex vivo. The molecule may be localized to the site of action, e.g. topical lotions, gels, creams and the like.

Stroke, traumatic brain injury, Alzheimer's disease, multiple sclerosis and other conditions affecting the blood brain barrier (BBB) may be treated with a Wnt surrogate molecule. Angiogenesis is critical to ensure the supply of oxygen and nutrients to many tissues throughout the body, and is especially important for the CNS as the neural tissue is extremely sensitive to hypoxia and ischemia. CNS endothelial cells which form the BBB differ from endothelial cells in non-neural tissue, in that they are highly polarized cells held together by tight junctions and express specific transporters. Wnt signaling regulates CNS vessel formation and/or function. Conditions in which the BBB is compromised can benefit from administration of the compounds of the invention, which can be systemic or localized e.g. by direct injection, intrathecal administration, implantation of sustained release formulations, and the like. In addition, Wnt signal is actively involved in neurogenesis and plays a role of neuroprotection following injury. The compositions of the present invention may also be used in treatment of spinal cord injuries, other spinal cord diseases, stroke, traumatic brain injuries, etc.

Wnt signals also play a role in angiogenesis. A Wnt surrogate molecule may benefit conditions where angiogenesis is beneficial, treatment of

myocardial infarction, coronary artery disease, heart failure, diabetic retinopathy,etc., and conditions from hereditary diseases. Methods for regeneration of these tissues benefit from administration of the compounds of the invention, which can be systemic or localized.

In certain embodiments, methods of the present invention promote tissue regeneration, e.g., in a tissue subjected to damage or tissue or cell reduction or loss. The loss or damage can be anything which causes the cell number to diminish, including diseases or injuries. For example, an accident, an autoimmune disorder, a therapeutic side-effect or a disease state could constitute trauma. Tissue regeneration increases the cell number within the tissue and preferably enables connections between cells of the tissue to be re-established, and more preferably the functionality of the tissue to be regained.

The terms "administering" or "introducing" or “providing”, as used herein, refer to delivery of a composition to a cell, to cells, tissues and/or organs of a subject, or to a subject. Such administering or introducing may take place in vivo, in vitro or ex vivo.

In particular embodiments, a pharmaceutical composition is administered parenterally, e.g., intravenously, orally, rectally, or by injection. In some embodiments, it is administered locally, e.g., topically or intramuscularly. In some embodiments, a composition is administered to target tissues, e.g., to bone, joints, ear tissue, eye tissue, gastrointestinal tract, skin, a wound site or spinal cord. Methods of the invention may be practiced in vivo or ex vivo. In some embodiments, the contacting of a target cell or tissue with a Wnt surrogate molecule is performed ex vivo, with subsequent implantation of the cells or tissues, e.g., activated stem or progenitor cells, into the subject. The skilled artisan can determine an appropriate site of and route of administration based on the disease or disorder being treated.

The dose and dosage regimen may depend upon a variety of factors readily determined by a physician, such as the nature of the disease or disorder, the characteristics of the subject, and the subject's history. In particular embodiments, the amount of a Wnt surrogate molecule administered or provided

to the subject is in the range of about 0.01 mg/kg to about 50 mg/kg, 0.1 mg/kg to about 500 mg/kg, or about 0.1 mg/kg to about 50 mg/kg of the subject's body weight.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof, e.g. reducing the likelihood that the disease or symptom thereof occurs in the subject, and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., causing regression of the disease. The therapeutic agent (e.g., a Wnt surrogate molecule) may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to complete loss of function in the affected tissues. The subject therapy will desirably be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease. In some embodiments, the subject method results in a therapeutic benefit, e.g., preventing the development of a disorder, halting the progression of a disorder, reversing the progression of a disorder, etc. In some embodiments, the subject method comprises the step of detecting that a therapeutic benefit has been achieved. The ordinarily skilled artisan will appreciate that such measures of therapeutic efficacy will be applicable to the particular disease being modified, and will recognize the appropriate detection methods to use to measure therapeutic efficacy.

Other embodiments relate, in part, to the use of the Wnt surrogate molecules disclosed herein to promote or enhance the growth or proliferation of cells, tissues and organoids, for example, by contacting cells or tissue with one

or more Wnt surrogate, optionally in combination with a Norrin or Rspondin polypeptide. In certain embodiments, the cells or tissue are contacted ex vivo, in vitro, or in vivo. Such methods may be used to generate cells, tissue or organoids for therapeutic use, e.g., to be transplanted or grafted into a subject. They may also be used to generate cells, tissue or organoids for research use. The Wnt surrogate molecules have widespread applications in non-therapeutic methods, for example in vitro research methods.

The invention provides a method for tissue regeneration of damaged tissue, such as the tissues discussed above, comprising administering a Wnt surrogate molecule to cells. The Wnt surrogate molecule may be administered directly to the cells in vivo, administered to a subject orally, intravenously, or by other methods known in the art, or administered to ex vivo cells. In some embodiments where the Wnt surrogate molecule is administered to ex vivo cells, these cells may be transplanted into a subject before, after or during administration of the Wnt surrogate molecule.

Wnt signaling is a key component of stem cell culture. For example, the stem cell culture media as described in WO2010/090513, WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 201 1; 141: 1762-1772) and Sato et al., 2009 (Nature 459, 262-5). The Wnt surrogate molecules disclosed herein are suitable alternatives to Rspondin for use in these stem cell culture media, or may be combined with Rspondin.

Accordingly, in one embodiment, the disclosure provides a method for enhancing the proliferation of stem cells comprising contacting stem cells with one or more Wnt surrogate molecules disclosed herein. In one embodiment, the disclosure provides a cell culture medium comprising one or more Wnt surrogate molecules disclosed herein. In some embodiments, the cell culture medium may be any cell culture medium already known in the art that normally comprises Wnt or Rspondin, but wherein the Wnt or Rspondin is replaced (wholly or partially) or supplemented by Wnt surrogate molecule(s) disclosed herein. For example, the culture medium may be as described in as described in WO2010/090513, WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 201 1; 141: 1762-

1772) and Sato et al., 2009 (Nature 459, 262-5), which are hereby incorporated by reference in their entirety.

Stem cell culture media often comprise additional growth factors. This method may thus additionally comprise supplying the stem cells with a growth factor. Growth factors commonly used in cell culture medium include epidermal growth factor (EGF, (Peprotech), Transforming Growth Factor-alpha (TGF-alpha, Peprotech), basic Fibroblast Growth Factor (bFGF, Peprotech), brain-derived neurotrophic factor (BDNF, R&D Systems), Hepatocyte Growth Factor (HGF) and Keratinocyte Growth Factor (KGF, Peprotech, also known as FGF7). EGF is a potent mitogenic factor for a variety of cultured ectodermal and mesodermal cells and has a profound effect on the differentiation of specific cells in vivo and in vitro and of some fibroblasts in cell culture. The EGF precursor exists as a membrane-bound molecule which is proteolytically cleaved to generate the 53-amino acid peptide hormone that stimulates cells. EGF or other mitogenic growth factors may thus be supplied to the stem cells. During culturing of stem cells, the mitogenic growth factor may be added to the culture medium every second day, while the culture medium is refreshed preferably every fourth day. In general, a mitogenic factor is selected from the groups consisting of: i) EGF, TGF-alpha, and KGF, ii) EGF, TGF-alpha, and FGF7; iii) EGF, TGF-alpha, and FGF; iv) EGF and KGF; v) EGF and FGF7; vi) EGF and a FGF; vii) TGF-alpha and KGF; viii) TGF-alpha, and FGF7; ix) or from TGF-alpha and a FGF. In certain embodiments, the disclosure includes a stem cell culture media comprising a Wnt surrogate molecule disclosed herein, e.g., optionally in combination with one or more of the growth factors or combinations thereof described herein.

These methods of enhancing proliferation of stem cells can be used to grow new organoids and tissues from stem cells, as for example described in WO2010/090513 WO2012/014076, Sato et al., 201 1 (GASTROENTEROLOGY 2011; 141: 1762-1772) and Sato et al., 2009 (Nature 459, 262-5).

In some embodiments, the Wnt surrogate molecules are used to enhance stem cell regeneration. Illustrative stem cells of interest include but are

not limited to: muscle satellite cells; hematopoietic stem cells and progenitor cells derived therefrom (U.S. Pat. No. 5,061 ,620); neural stem cells (see Morrison et al. (1999) Cell 96: 737-749); embryonic stem cells; mesenchymal stem cells; mesodermal stem cells; liver stem cells; adipose-tissue derived stem cells, etc.

Other embodiments of the present invention relate, in part, to diagnostic applications for detecting the presence of cells or tissues expressing one or more Fzd receptors or LRP5 or LRP6 receptors. Thus, the present disclosure provides methods of detecting one or more Fzd receptor or LRP5 or LRP6 receptor in a sample, such as detection of cells or tissues expressing Fzd1. Such methods can be applied in a variety of known detection formats, including, but not limited to immunohistochemistry (IHC), immunocytochemistry (ICC), *in situ* hybridization (ISH), whole-mount *in situ* hybridization (WISH), fluorescent DNA *in situ* hybridization (FISH), flow cytometry, enzyme immuno-assay (EIA), and enzyme linked immuno-assay (ELISA), e.g., by detecting binding of a Wnt surrogate molecule.

ISH is a type of hybridization that uses a labeled complementary DNA or RNA strand (*i.e.*, primary binding agent) to localize a specific DNA or RNA sequence in a portion or section of a cell or tissue (*in situ*), or if the tissue is small enough, the entire tissue (whole mount ISH). One having ordinary skill in the art would appreciate that this is distinct from immunohistochemistry, which localizes proteins in tissue sections using an antibody as a primary binding agent. DNA ISH can be used on genomic DNA to determine the structure of chromosomes. Fluorescent DNA ISH (FISH) can, for example, be used in medical diagnostics to assess chromosomal integrity. RNA ISH (hybridization histochemistry) is used to measure and localize mRNAs and other transcripts within tissue sections or whole mounts.

In various embodiments, the Wnt surrogate molecules described herein are conjugated to a detectable label that may be detected directly or indirectly. In this regard, an antibody "conjugate" refers to a Wnt surrogate molecule that is covalently linked to a detectable label. In the present invention, DNA probes, RNA probes, monoclonal antibodies, antigen-binding fragments

thereof, and antibody derivatives thereof, such as a single-chain-variable-fragment antibody or an epitope tagged antibody, may all be covalently linked to a detectable label. In “direct detection”, only one detectable antibody is used, *i.e.*, a primary detectable antibody. Thus, direct detection means that the antibody that is conjugated to a detectable label may be detected, per se, without the need for the addition of a second antibody (secondary antibody).

A "detectable label" is a molecule or material that can produce a detectable (such as visually, electronically or otherwise) signal that indicates the presence and/or concentration of the label in a sample. When conjugated to an antibody, the detectable label can be used to locate and/or quantify the target to which the specific antibody is directed. Thereby, the presence and/or concentration of the target in a sample can be detected by detecting the signal produced by the detectable label. A detectable label can be detected directly or indirectly, and several different detectable labels conjugated to different specific-antibodies can be used in combination to detect one or more targets.

Examples of detectable labels, which may be detected directly, include fluorescent dyes and radioactive substances and metal particles. In contrast, indirect detection requires the application of one or more additional antibodies, *i.e.*, secondary antibodies, after application of the primary antibody. Thus, the detection is performed by the detection of the binding of the secondary antibody or binding agent to the primary detectable antibody. Examples of primary detectable binding agents or antibodies requiring addition of a secondary binding agent or antibody include enzymatic detectable binding agents and hapten detectable binding agents or antibodies.

In some embodiments, the detectable label is conjugated to a nucleic acid polymer which comprises the first binding agent (*e.g.*, in an ISH, WISH, or FISH process). In other embodiments, the detectable label is conjugated to an antibody which comprises the first binding agent (*e.g.*, in an IHC process).

Examples of detectable labels which may be conjugated to Wnt surrogate molecules used in the methods of the present disclosure include

fluorescent labels, enzyme labels, radioisotopes, chemiluminescent labels, electrochemiluminescent labels, bioluminescent labels, polymers, polymer particles, metal particles, haptens, and dyes.

Examples of fluorescent labels include 5-(and 6)-carboxyfluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid, fluorescein isothiocyanate, rhodamine, tetramethylrhodamine, and dyes such as Cy2, Cy3, and Cy5, optionally substituted coumarin including AMCA, PerCP, phycobiliproteins including R-phycoerythrin (RPE) and allophycoerythrin (APC), Texas Red, Princeton Red, green fluorescent protein (GFP) and analogues thereof, and conjugates of R-phycoerythrin or allophycoerythrin, inorganic fluorescent labels such as particles based on semiconductor material like coated CdSe nanocrystallites.

Examples of polymer particle labels include micro particles or latex particles of polystyrene, PMMA or silica, which can be embedded with fluorescent dyes, or polymer micelles or capsules which contain dyes, enzymes or substrates.

Examples of metal particle labels include gold particles and coated gold particles, which can be converted by silver stains. Examples of haptens include DNP, fluorescein isothiocyanate (FITC), biotin, and digoxigenin. Examples of enzymatic labels include horseradish peroxidase (HRP), alkaline phosphatase (ALP or AP), β-galactosidase (GAL), glucose-6-phosphate dehydrogenase, β-N-acetylglucosamimidase, β-glucuronidase, invertase, Xanthine Oxidase, firefly luciferase and glucose oxidase (GO). Examples of commonly used substrates for horseradishperoxidase include 3,3'-diaminobenzidine (DAB), diaminobenzidine with nickel enhancement, 3-amino-9-ethylcarbazole (AEC), Benzidine dihydrochloride (BDHC), Hanker-Yates reagent (HYR), Indophane blue (IB), tetramethylbenzidine (TMB), 4-chloro-1-naphtol (CN), .alpha.-naphtol pyronin (.alpha.-NP), o-dianisidine (OD), 5-bromo-4-chloro-3-indolylphosp- hate (BCIP), Nitro blue tetrazolium (NBT), 2-(p-iodophenyl)-3-p-nitropheny- l-5-phenyl tetrazolium chloride (INT), tetranitro blue tetrazolium

(TNBT), 5-bromo-4-chloro-3-indoxyl-beta-D-galactoside/ferro-ferricyanide (BCIG/FF).

Examples of commonly used substrates for Alkaline Phosphatase include Naphthol-AS-B 1-phosphate/fast red TR (NABP/FR), Naphthol-AS-MX-phosphate/fast red TR (NAMP/FR), Naphthol-AS-B1-phosphate/- fast red TR (NABP/FR), Naphthol-AS-MX-phosphate/fast red TR (NAMP/FR), Naphthol-AS-B1-phosphate/new fuschin (NABP/NF), bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT), 5-Bromo-4-chloro-3-indolyl-b-- d-galactopyranoside (BCIG).

Examples of luminescent labels include luminol, isoluminol, acridinium esters, 1,2-dioxetanes and pyridopyridazines. Examples of electrochemiluminescent labels include ruthenium derivatives. Examples of radioactive labels include radioactive isotopes of iodide, cobalt, selenium, tritium, carbon, sulfur and phosphorous.

Detectable labels may be linked to the antibodies described herein or to any other molecule that specifically binds to a biological marker of interest, *e.g.*, an antibody, a nucleic acid probe, or a polymer. Furthermore, one of ordinary skill in the art would appreciate that detectable labels can also be conjugated to second, and/or third, and/or fourth, and/or fifth binding agents or antibodies, etc. Moreover, the skilled artisan would appreciate that each additional binding agent or antibody used to characterize a biological marker of interest may serve as a signal amplification step. The biological marker may be detected visually using, *e.g.*, light microscopy, fluorescent microscopy, electron microscopy where the detectable substance is for example a dye, a colloidal gold particle, a luminescent reagent. Visually detectable substances bound to a biological marker may also be detected using a spectrophotometer. Where the detectable substance is a radioactive isotope detection can be visually by autoradiography, or non-visually using a scintillation counter. See, *e.g.*, Larsson, 1988, Immunocytochemistry: Theory and Practice, (CRC Press, Boca Raton, Fla.); Methods in Molecular Biology, vol. 80 1998, John D. Pound (ed.) (Humana Press, Totowa, N.J.).

The invention further provides kits for detecting one or more Fzd or LRP5/6 receptor or cells or tissues expressing one or more Fzd or LRP5/6 receptors in a sample, wherein the kits contain at least one antibody, polypeptide, polynucleotide, vector or host cell as described herein. In certain embodiments, a kit may comprise buffers, enzymes, labels, substrates, beads or other surfaces to which the antibodies of the invention are attached, and the like, and instructions for use.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

EXAMPLES

Example 1

Illustrative Wnt surrogate Molecule Formats

Wnt surrogates having a variety of different formats disclosed herein were made. These included the following illustrative formats, each comprising a binding domain ("binder") that binds to one or more Fzd receptor and a binding domain ("binder") that binds to an LRP5 and/or LRP6 receptor.

As shown in Fig. 1A, if a binder to one receptor is a Fab and to the other receptor is a Nab or scFv, they can be put together in several different configurations. In certain instances, the Fab binder first can be reformatted to a full IgG format, then the Nab binder can be fused to any of the 4 available termini of the IgG. For example, the Nab can be fused to the N-terminus of the IgG light chain (LC, the fusion will be referred to as NL, shown in top left), N-terminus of the IgG heavy chain (HC, the fusion will be referred to as NH, shown in top right),

the C-terminus of LC (the fusion will be referred to as CL, shown in middle right), and the C-terminus of HC (the fusion will be referred to as CH, shown in middle left). The linkers and the length of the linkers between the IgG and the Nab can be varied. These four formats are bispecific and bivalent, they are bivalent binders toward each of the receptors. An alternative way to put the two binders together is the Hetero-Ig format where the Fab binder is presented as a half antibody, and the Nab is fused to the N-terminus of an Fc (shown in lower middle). The two halves may be brought together by mutations in the CH3 domain (such as the knobs-into-holes) that favor the formation of the heterodimer. The linker and its length between the Nab binder and the Fc can be varied. This format would be bispecific but monovalent toward each receptor. The Nab part of any of the formats described in this example can also be replaced by scFv fragments of binders as well.

As shown in Fig. 1B, if a binder to one receptor is a Fab and to the other receptor is also a Fab, they can be put together in several different configurations. In one approach, one Fab binder is first reformatted to a full IgG format (shown in top). The second Fab binder can be fused to the N-terminus of the IgG. The two HC can be fused together with a linker in between. The LCs can be fused or unfused. The linker and its length can be varied. This format is a bispecific and bivalent format. Alternatively, the second Fab binder LC can be fused to the HC of the IgG with a linker of various length in between. The second Fab binder HC can be fused or unfused to the LC of the IgG. A variation of this format has been called Fabs-in-tandem IgG (or FIT-Ig). In another approach, the two binders can be brought together as a Hetero-Ig by mutations in the CH3 domain that favors the heterodimer assembly, the two arms will each bind to one receptor (shown in bottom). This format is a bispecific and monovalent binder.

As shown in Fig. 1C, if a binder to one receptor is a Nab and to the other receptor is also a Nab, they can be put together in several different configurations. In the bispecific bivalent formats, in certain instances, the two Nab binders can be fused together in tandem (shown in top row) or fused to the two-different ends of the Fc (shown in middle row). The linker and its length

between Nab and Nab or Nab and Fc can be varied. Alternatively, the two Nabs can be assembled together as Hetero-Ig to generate a bispecific and monovalent format (shown in bottom row). Similar to Fig 1A, the Nab domains here can also be replaced by an scFv domain of a binder. In all the examples, the Nab and scFv can be mixed in certain combinations as well.

As shown in Fig. 1D, the binders against Fzd and LRP can also be linked together in a diabody (or DART) configuration. The diabody can also be in a single chain configuration. If the diabody is fused to an Fc, this will create a bivalent bispecific format. Without fusion to Fc, this would be a monovalent bispecific format.

A number of Wnt surrogates representing different configurations were produced. These included the Wnt surrogates described in Table 3. These illustrative Wnt surrogates include one, two or three polypeptides, the sequences of which are provided as Sequence 1, Sequence 2, and/or Sequence 3. The sequence may include a leader peptide sequence, a Nab sequence, a linker, and/or a heavy or light chain sequence. Annotated sequences are provided in FIG. 19, in which the leader peptide sequence is italicized, the linker sequence is underlined, the Nab sequence is shown in bold, and the remaining sequence is the heavy chain or light chain sequence. The Fzd binder IDs and LRP binder IDs correspond to the clone numbers provided in Tables 1A-B and 2A-B for various Fzd binding or LRP5/6 binding antibodies or antigen-binding fragments thereof.

The Wnt surrogates beginning with “R2M3” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 001S-A04. The first six Wnt surrogates beginning with “18R5” in Table 3 include different LRP6 binding domains fused to the N-terminus of the anti-Fzd antibody or antigen-binding fragment thereof named 18R5. The Wnt surrogates beginning with “1R” include the anti-LRP6 antibody of antigen-binding fragment thereof named “009S-E04” fused to the N-terminus of different anti-Fzd antibodies or antigen-binding fragment thereof. For “R2M3-26CH,” the LRP6 binding region is fused to the C-terminus of the Fzd binding region. For “R2M3-26NH,” the LRP6 binding region

is fused to the N-terminus of the Fzd binding region. For “R2M3-26CL,” the LRP6 binding region is fused to the C-terminus of the Fzd binding region. For “R2M3-26NL,” the LRP6 binding region is fused to the N-terminus of the Fzd binding region. For “R2M3-26Fab” and “R2M3-32Fab,” the LRP6 binding region is fused to the N-terminus of the Fzd binding region. For “Hetero-Ig,” the LRP6 binding region is fused to the N-terminus of human Fc_hole, and paired with Fzd binder light chain and heavy chain human IgG1_knob. The Wnt surrogates beginning with “17SB9” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 017S-B09. The Wnt surrogates beginning with “1R-C07” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 001S-B03. The Wnt surrogates beginning with “R2M13” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 004S-G06. The Wnt surrogates beginning with “3SD10” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 003S-D10. The Wnt surrogates beginning with “4SD1” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 004S-D01. The Wnt surrogates beginning with “14SB6” include different LRP6 binding domains fused to the N-terminus of a light chain region of the anti-Fzd antibody or antigen-binding fragment thereof named 014S-B06.

Table 3. Wnt Surrogate Sequences

Name	Fzd binder ID	LRP binder ID	Sequence 1 SEQ ID NO	Sequence 2 SEQ ID NO	Sequence 3 SEQ ID NO
R2M3-23	001S-A04	009S-B04	89	95	N/A
R2M3-26	001S-A04	009S-E04	90	95	N/A

R2M3-28	001S-A04	009S-G04	91	95	N/A
R2M3-29	001S-A04	009S-H04	92	95	N/A
R2M3-31	001S-A04	013S-G04	93	95	N/A
R2M3-32	001S-A04	013S-H04	94	95	N/A
18R5-5	18R5	008S-G01	96	104	N/A
18R5-7	18R5	008S-C02	97	104	N/A
18R5-8	18R5	008S-D02	98	104	N/A
18R5-9	18R5	008S-E02	99	104	N/A
18R5-26	18R5	009S-E04	100	104	N/A
18R5-28	18R5	009S-G04	101	104	N/A
18R5-31	18R5	013S-G04	102	104	N/A
18R5-32	18R5	013S-H04	103	104	N/A
1R-B05-26	001S-E02	009S-E04	105	111	N/A
1R-C01-26	001S-B01	009S-E04	106	112	N/A
1R-C07-26	001S-B03	009S-E04	107	113	N/A

1R-E06-26	001S-H02	009S-E04	108	114	N/A
1R-G05-26	001S-G02	009S-E04	109	115	N/A
1R-G06-26	001S-A03	009S-E04	110	116	N/A
R2M3-26 CH	001S-A04	009S-E04	125	117	N/A
R2M3-26 NH	001S-A04	009S-E04	125	118	N/A
R2M3-26 CL	001S-A04	009S-E04	119	2254	N/A
R2M3-26 NL	001S-A04	009S-E04	120	2254	N/A
R2M3-26 Fab	001S-A04	009S-E04	120	122	N/A
R2M3-26F(ab')2	001S-A04	009S-E04	120	2252	
R2M3-32 Fab	001S-A04	013S-H04	123	122	N/A
R2M3-26 Hetero-Ig	001S-A04	009S-E04	125	126	127
26-17SB9	017S-B09	009S-E04	128	N/A	N/A
26:Fc:17SB9-criss-cross	017S-B09	009S-E04	2192	2193	N/A
26:5:17SB9:Fc	017S-B09	009S-E04	2194	N/A	N/A
26:10:17SB9:Fc	017S-B09	009S-E04	2195	N/A	N/A

26:15:17SB9:Fc	017S-B09	009S-E04	2196	N/A	N/A
17SB9:Fc:26	017S-B09	009S-E04	2197	N/A	N/A
26:Fc:26+17SB9:Fc:17SB9	017S-B09	009S-E04	2198	2199	N/A
1R-C07-3	001S-B03	008S-D01	134	113	N/A
1R-C07-36	001S-B03	013S-D05	135	113	N/A
R2M13-3	004S-G06	008S-D01	136	153	N/A
R2M13-26	004S-G06	009S-E04	137	153	N/A
R2M13-36	004S-G06	013S-D05	138	153	N/A
R2M3-3	001S-A04	008S-D01	139	2254	N/A
R2M3-36	001S-A04	013S-D05	140	2254	N/A
3SD10-3	003S-D10	008S-D01	141	154	N/A
3SD10-26	003S-D10	009S-E04	142	154	N/A
3SD10-36	003S-D10	013S-D05	143	154	N/A
4SD1-3	004S-D01	008S-D01	144	155	N/A
4SD1-26	004S-D01	009S-E04	145	155	N/A

4SD1-36	004S-D01	013S-D05	146	155	N/A
14SB6-3	014S-B06	008S-D01	147	156	N/A
14SB6-26	014S-B06	009S-E04	148	156	N/A
14SB6-36	014S-B06	013S-D05	149	156	N/A
R2M9-3	003S-E07	008S-D01	150	157	N/A
R2M9-26	003S-E07	009S-E04	151	157	N/A
R2M9-36	003S-E07	013S-D05	152	157	N/A
18R5:5:1115.3:Fc	18R5	1115.3	2200	N/A	N/A
18R5:10:1115.3:Fc	18R5	1115.3	2201	N/A	N/A
18R5:15:1115.3:Fc	18R5	1115.3	2202	N/A	N/A
1115.3:5:18R5:Fc	18R5	1115.3	2203	N/A	N/A
1115.3:10:18R5:Fc	18R5	1115.3	2204	N/A	N/A
1115.3:15:18R5:Fc	18R5	1115.3	2205	N/A	N/A
18R5:5:YW211.31.57:Fc	18R5	YW211.31.57	2206	N/A	N/A

18R5:10:YW211.31.57:Fc	18R5	YW211.31.57	2207	N/A	N/A
18R5:15:YW211.31.57:Fc	18R5	YW211.31.57	2208	N/A	N/A
YW211.31.57:5:18R5:Fc	18R5	YW211.31.57	2209	N/A	N/A
YW211.31.57:10:18R5:Fc	18R5	YW211.31.57	2210	N/A	N/A
YW211.31.57:15:18R5:Fc	18R5	YW211.31.57	2211	N/A	N/A
18R5:Fc:1115.3	18R5	1115.3	2212	N/A	N/A
1115.3:Fc:18R5	18R5	1115.3	2213	N/A	N/A
18R5:Fc:YW211.31.57	18R5	YW211.31.57	2250	N/A	N/A
YW211.31.57:Fc:18R5	18R5	YW211.31.57	2267	N/A	N/A
421.1-R2M3 cp	001S-A04	421.1	2214	2215	2216
1RC07:5:10SA7 cp	1RC07	10SA7	2217	2218	2219
1RC07:10:10SA7 cp	1RC07	10SA7	2217	2218	2222
1RC07:15:10SA7 cp	1RC07	10SA7	2217	2218	2225

1RC07:5:10SG7 cp	1RC07	10SG7	2217	2227	2228
1RC07:10:10SG7 cp	1RC07	10SG7	2217	2227	2231
1RC07:15:10SG7 cp	1RC07	10SG7	2217	2227	2234
10SG7:5:1RC07 cp	1RC07	10SG7	2227	2217	2237
10SG7:10:1RC07 cp	1RC07	10SG7	2227	2217	2240
10SG7:15:1RC07 cp	1RC07	10SG7	2227	2217	2243
1RC07:5:10SA7 L->H	1RC07	10SA7	2244	2245	2246
1115.3:5:R2M3 L->H	001S-A04	1115.3	2247	125	2248
1115.3:10:R2M3 L->H	001S-A04	1115.3	2247	125	2249
10SG11-1RC07	1RCO7	10SG11	2252	2253	N/A
18R5:5:1115.3:His	18R5	1115.3	2255	N/A	N/A
18R5:10:1115.3:His	18R5	1115.3	2256	N/A	N/A
18R5:15:1115.3:His	18R5	1115.3	2257	N/A	N/A
1115.3:5:18R5:His	18R5	1115.3	2258	N/A	N/A
1115.3:10:18R5:His	18R5	1115.3	2259	N/A	N/A

1115.3:15:18R5:His	18R5	1115.3	2260	N/A	N/A
18R5:5:YW211.31.57:His	18R5	YW211.31.57	2261	N/A	N/A
18R5:10:YW211.31.57:His	18R5	YW211.31.57	2262	N/A	N/A
18R5:15:YW211.31.57:His	18R5	YW211.31.57	2263	N/A	N/A
YW211.31.57:5:18R5:His	18R5	YW211.31.57	2264	N/A	N/A
YW211.31.57:10:18R5:His	18R5	YW211.31.57	2265	N/A	N/A
YW211.31.57:15:18R5:His	18R5	YW211.31.57	2266	N/A	N/A

Example 2

Characterization of a Wnt Surrogate Molecule, R2M3-26

The R2M3-26 molecule consists of a Fzd binder (R2M3) and a LRP6 binder (26). The LRP6 binder 26 was fused to the N-terminus of R2M3 LC with a 5-amino acid linker as depicted in Fig. 2A. R2M3 was in the form of an IgG. The protein was purified by Protein A affinity column followed by a size-exclusion-chromatography (SEC) step. The absorbance trace from the SEC and the SDS-PAGE gels of the SEC fractions were shown in Fig. 2B. The ability of R2M3-26 to activate canonical Wnt signaling was tested in a Wnt responding 293 reporter cell line (293STF). The 293STF reporter activity traces across the SEC

fractions were shown in Fig. 2B, the peak of the reporter activity correlated with the peak of the proteins. The peak fraction was further characterized by a dose response in 293STF cells in the absence and presence of R-spondin (Fig. 2D). R2M3-26 induced reporter activity in a dose dependent manner and was enhanced by the presence of R-spondin similar to a natural Wnt ligand, while R2M3 IgG alone without the attachment of the LRP binding arm did not induce reporter activity. The ability of R2M3-26 to interact with its target, a Fzd1 ECD, was performed in Octet interaction assay (Fig. 2C), and the results showed that the fusion of the LRP6 binding arm, 26, did not affect R2M3 interaction with its target, Fzd.

Example 3

Characterization of a Wnt Surrogate Molecule, R2M3-32

The R2M3-32 molecule consists of a Fzd binder (R2M3) and a LRP6 binder (32). The LRP6 binder 32 was fused to the N-terminus of R2M3 LC with a 5-amino acid linker as depicted in Fig. 3A. R2M3 was in the form of an IgG. The protein was purified by Protein A affinity column followed by a size-exclusion-chromatography (SEC) step. The absorbance trace from the SEC and the SDS-PAGE gels of the SEC fractions were shown in Fig. 3B. The ability of R2M3-32 to activate canonical Wnt signaling was tested in a Wnt responding 293 reporter cell line (293STF). The 293STF reporter activity traces across the SEC fractions were shown in Fig. 3B, the peak of the reporter activity correlated with the peak of the proteins. The peak fraction was further characterized by a dose response in 293STF cells in the absence and presence of R-spondin (Fig. 3D). R2M3-32 induced reporter activity in a dose dependent manner and was enhanced by the presence of R-spondin, while R2M3 IgG alone without the attachment of the LRP binding arm did not induce reporter activity. The ability of R2M3-32 to interact with its target, a Fzd1 extracellular domain (ECD), was performed in Octet interaction assay (Fig. 3C). The results showed that the fusion of the LRP6 binding arm, 32, did not affect R2M3 interaction with its target, Fzd.

Example 4

R2M3-26 and R2M3-32 Activities Can Be Inhibited by Soluble Fzd ECD and by R2M3 IgG Alone without the LRP Binding Arm

The ability of soluble Fzd extracellular domain (ECD) or R2M3 IgG alone to inhibit Wnt surrogates was determined using the 293STF reporter assay. The Fzd1ECD-Fc or R2M3 IgG was titrated into the 293STF reporter assay, at a fixed concentration of R2M3-26 or R2M3-32. In a dose dependent manner, both Fzd1ECD-Fc and R2M3 IgG inhibited R2M3-26 (Fig. 4A) and R2M3-32 (Fig. 4B) induced reporter signaling, while the negative control molecule, Fc alone had no impact.

Example 5

Characterization of R2M3-LRP6 Binder Fusions in 293, Huh7, A375, BNL.CL2 Wnt Dependent Reporter Assays

The Fzd binder, R2M3, was fused to additional LRP6 binders, 23, 25, 26, 27, 28, 29, 31, 32, 33, and 36. The LRP6 binders were Nab and were fused to the N-terminus of R2M3 LC with a 5-amino acid linker. These proteins were purified by Protein A affinity column followed by a SEC step. The fusion proteins were tested in Wnt dependent reporter assays in 293, Huh7, A375, and BNL.CL2 cell lines, and activated Wnt signaling to various levels. R2M3 was also fused to two non-LRP6 binder Nabs, 24 and 34, in the same format as the Lrp6 binders. These two non-binders displayed no activity in Wnt dependent 293 reporter assay (Fig. 5), suggesting that Wnt activities observed with R2M3 fusions to 23, 25, 26, 27, 28, 29, 31, 32, 33, and 36 are dependent on the presence of both Fzd and Lrp mimicking the nature ligand function.

Example 6

Characterization of 18R5-LRP6 Binder Fusions in 293, A375, and BNL.CL2 Wnt Dependent Reporter Assays

The Fzd binder, 18R5, was fused to LRP6 binders, 26, 28, 31, 32. The LRP6 binders were Nab and were fused to the N-terminus of 18R5 LC with a 5-amino acid linker. These proteins were purified by Protein A affinity column followed by a SEC step. The fusion proteins were tested in Wnt dependent reporter assays in 293, A375, and BNL.CL2 cell lines and demonstrated ability to activate Wnt signaling (Fig. 6).

Example 7

Characterization of 18R5-LRP5 Binder Fusions in 293 Wnt Dependent Reporter Assays

The Fzd binder, 18R5, was fused to LRP5 binders, 5, 7, 8, 9. The LRP5 binders were Nab and were fused to the N-terminus of 18R5 LC with a 5-amino acid linker. These proteins were purified by Protein A affinity column followed by a SEC step. The fusion proteins were tested in a Wnt dependent reporter assays in 293 cells and were able to activate Wnt signaling (Fig. 7).

Example 8

Characterization of Various Fzd binders-LRP6 Binder 26 Fusions in 293 Wnt Dependent Reporter Assays

The various Fzd binders, 1R-B05, 1R-C01, 1R-C07, 1R-E01, 1R-E06, 1R-G05, 1R-G06, 1R-H04, in IgG format were fused to LRP6 binders, 26. The LRP6 binder Nab was fused to the N-terminus of various Fzd binder LC with a 5-amino acid linker. These proteins were purified by Protein A affinity column followed by a SEC step. The SDS-PAGE gel analysis of the SEC peak fractions were shown in Fig. 8A. The fusion proteins were tested in a Wnt dependent reporter assays in 293 cells in the presence of Rspo and were able to activate Wnt signaling (Fig. 8B).

Example 9

SAR Analysis of the IgG-Nab Fusion Format

SAR analysis of the IgG-Nab fusions was performed by rotating the attachment location of the Nab to the different termini of the IgG HC or LC as depicted in Fig. 1A. CH indicates attaching the Nab to the C-terminus of heavy chain; NH indicates attaching the Nab to the N-terminus of heavy chain; CL indicates attaching the Nab to the C-terminus of light chain; NL indicates attaching the Nab to the N-terminus of light chain. Three pairs of IgG-Nab fusions SARs were shown, the pairs were between R2M3 and 26, between R2M3 and 32, and between 18R5 and 26. The assays were performed on Wnt responsive 293 reporter cells in the presence of Rspo and activated Wnt signaling to various levels (Fig. 9). These results demonstrate that the attachment location of the fusion and the geometry between the Fzd and LRP binding domains play roles in the ability of the Wnt surrogates to activate Wnt signaling.

Example 10

Characterization of R2M3-26 in the Fab Format

The molecule R2M3-26 Fab consists of a Fzd binder (R2M3) and a LRP6 binder (26). The LRP6 binder 26 was fused to the N-terminus of R2M3 LC with a 5-amino acid linker as depicted in Fig. 10A. R2M3 was in the form of a Fab. The protein was purified by Ni-NTA affinity column followed by a size-exclusion-chromatography (SEC) step. The absorbance trace from the SEC and the SDS-PAGE gels of the SEC fractions are shown in Fig. 10B. The ability of R2M3-26 as a Fab format to activate canonical Wnt signaling was tested in a Wnt responding 293 reporter cells (293STF). The 293STF reporter activity traces across the SEC fractions are shown in Fig. 10B. Unlike when R2M3 was in the IgG format shown in Fig. 2, the peak of the reporter activity from the R2M3 in the Fab format did not correlate with the peak of the proteins. These results suggest that R2M3-26 fusion in the Fab format is ineffective in inducing canonical Wnt signaling as detected by a reporter assay.

Example 11

Characterization of R2M3-32 in the Fab Format

The molecule R2M3-32 Fab consists of a Fzd binder (R2M3) and a LRP6 binder (32). The Lrp6 binder 32 was fused to the N-terminus of R2M3 LC with a 5-amino acid linker as depicted in Fig. 11A. R2M3 was in the form of a Fab. The protein was purified by Ni-NTA affinity column followed by a size-exclusion-chromatography (SEC) step. The absorbance trace from the SEC and the SDS-PAGE gels of the SEC fractions were shown in Fig. 11B. The ability of R2M3-32 as a Fab format to activate canonical Wnt signaling was tested in a Wnt responding 293 reporter cells (293STF). The 293STF reporter activity traces across the SEC fractions are shown in Fig. 11B. Unlike when R2M3 was in the IgG format shown in Fig. 3, the peak of the reporter activity from the R2M3 in the Fab format did not correlate with the peak of the proteins. These results suggest that R2M3-32 fusion in the Fab format is ineffective in inducing canonical Wnt signaling as detected by a reporter assay.

Example 12

Characterization of R2M3-26 in the HeteroIg Format

The molecule R2M3-26 HeteroIg consists of a Fzd binder (R2M3) and a LRP6 binder (26) as depicted in Fig. 12A and described in Fig. 1A. The protein was purified by Protein A affinity column followed by a size-exclusion-chromatography (SEC) step. The peak fraction from the SEC column was tested in a dose response in the Wnt responsive 293STF reporter cells in the absence or presence of R-spondin (Fig. 12B). Compared to R2M3-26 in the IgG format (as described in Fig. 2), R2M3-26 HeteroIg was ineffective in inducing canonical Wnt signaling as detected in the 293 reporter assay.

Example 13

Characterization of 26-17SB9 in the Nab-Nab Format

The molecule 26-17SB9 Nab-Fc-Nab consists of a LRP6 binder (26) and a Fzd binder (17SB9) as depicted in Fig. 13A and described in Fig. 1C. The protein was purified by Protein A affinity column followed by a size-exclusion-chromatography (SEC) step. The peak fraction from the SEC column

was tested in a dose response in the Wnt responsive 293STF reporter cells in the absence or presence of R-spondin (Fig. 13B). 26-17SB9 in the Nab-Fc-Nab format induced canonical Wnt signaling as detected in the 293 reporter assay.

Additional combinations of 26 and 17SB9 were also constructed (Fig. 13C) and tested in 293 reporter assays. As shown in Fig. 13D and 13E, these various combinations where 26 and 17SB9 were arranged in different tandem formats or on different ends of the Fc fragment all activated Wnt signaling to various levels in the presence of 20nM R-spondin

Example 14

Characterization of 18R5-LRP6 Binder Fusions in Tandem scFv Formats

The Fzd binder 18R5, the LRP6E1E2 binder 1115.3 (as described in PCT Publication WO2009/064944), and the LRP6E3E4 binder YW211.31.57 (as described in PCT Publication WO2011/119661) were converted into scFv format. 1115.3_scFv or YW211.31.57_scFv is assembled to the N-terminus of 18R5_scFv with a 5, 10 or 15 - amino acid linker and 18R5_scFv C-terminus is fused to a human Fc domain. In another set of examples, 1115.3_scFv or YW211.31.57_scFv is assembled to the C-terminus of 18R5 scFv with a 5, 10 or 15 - amino acid linker and human Fc domain is fused to the C-terminus of LRP binders. These formats are depicted in Fig. 14G left panel. In another example, 18R5_scFv and LRP binder, 1115.3_scFv, or LRP binder, YW211.31.57_scFv were fused to the two ends of a human Fc domain (as depicted in Fig. 14G right panel). These proteins were purified by Protein A affinity column followed by a SEC step.

The fusion proteins were tested in a Wnt dependent reporter assays in 293 cells. 18R5_scFv-1115.3_scFv-Fc and 1115.3_scFv-18R5_scFv-Fc with 5, 10 or 15-mer linker were able to activate Wnt signaling (Fig. 14A and 14B). 18R5_scFv-YW211.31.57_scFv-Fc and YW211.31.57_scFv-18R5_scFv-Fc with different linkers activated Wnt signaling (Fig. 14C and 14D). In addition, 18R5_scFv and 1115.3_scFv or YW211.31.57 fused to the two ends of Fc also activated Wnt signaling (Fig. 14E and F). While all of these scFv formats

activated Wnt signaling, potency and overall maximal efficacy may differ depends on the binder combination, linker length, and relative orientation.

In another example, 1115.3_scFv or YW211.31.57_scFv is assembled to the N-terminus or C-terminus of 18R5_scFv with a 5, 10 or 15 - amino acid linker without the further fusion of Fc, to create a bispecific but monovalent binding to each of Fzd or LRP. As shown in Fig. 14H, the 1115.3_scFv and 18R5_scFv fusions were in effective in activating Wnt signaling in the presence of 20nM R-spondin in 293 reporter cells.

Example 15

Generation of Wnt Surrogate Molecules in the Fab-IgG Format

Wnt mimetic or surrogate molecules can be generated in various formats when both the FZD and LRP binders are Fabs. Various approaches, such as charge paring, “knobs-in-holes”, crossover of the heavy and light chains of Fabs, can be employed to ensure proper heavy and light chain pairing. Two examples are given below.

1. Charge-pairing (cp) approach for Fab-on-IgG format: The heavy chain (VH-CH1) domain of an anti-LRP6 Fab, through a linker of 5, 10, or 15-mer amino acids, was fused in tandem with the N-terminus of the heavy chain (VH-CH1-CH2-CH3) of an anti-FZD binder. Both VH-CH1 domains of anti-LRP6 and anti-FZD contain three amino acid mutations (Q39D, Q105D, S183K in the anti-LRP6 Fab; Q39K, Q105K, S183E in anti-FZD Fab) each for proper paring with their own partner light chains, which also contain three complementary amino acid mutations (Q38K, A/S43K, S176E in anti-LRP6 light chain; Q38D, A/S43D, S176K in the anti-FZD light chain). The order of the anti-LRP6 and anti-FZD Fabs could be reversed, where the anti-FZD binder is a Fab and is fused to anti-LRP binder which is in IgG format (Fig. 15A).

2. HC-LC cross over approach for Fab-on-IgG format: The light chain (VL-CL) domains of anti-LRP6 binder was, through a linker of 5, 10, or 15-mer amino acids, fused in tandem with the N-terminus of the heavy chain

(VH-CH1-CH2-CH3) of an anti-FZD binder. The second construct was VH-CH1 of the anti-LRP6 binder and the third construct was VL-CL of the anti-FZD binder. Similar to the example above, the order of the anti-LRP6 and the anti-FZD binders could be reversed, where anti-FZD binder Fab is fused to the N-terminus of the anti-LRP binder which is in IgG format (Fig. 15A).

Several different pairs of LRP and FZD binders were assembled in these formats and tested in a Wnt responding 293 reporter cell line (293STF). As an example, the anti LRP6E1E2 binder 421.1 (as described in PCT Publication WO2009/064944) was fused to the N-terminus of anti-FZD binder, R2M3, using the charge paring approach to generate, 421.1-R2M3 cp. 421.1-R2M3 cp dose-dependently activated Wnt signaling in the 293 reporter assay (Fig. 15B). An anti-FZD binder, 1RC07, was fused to the N-terminus of an anti-LRP binder, 10SA7, with 5, 10, or 15-mer linkers. All three fusion proteins activated Wnt signaling (Fig. 15C). The anti-FZD binder, 1RC07, was further fused with the anti-LRP binder, 10SG7, either with 1RC07 in the Fab format fused to the N-terminus of 10SG7 in IgG format, or in the reverse order where 10SG7 as Fab fused to the N-terminus of 1RC07 as IgG with either 5, 10, or 15-mer amino acid linkers. All fusion molecules activated Wnt signaling while some preference of orientation and linker length was observed (Fig. 15D and 15E).

The HC-LC crossover Fab-IgG format was also tested. The anti-FZD binder 1RC07 LC was fused to the N-terminus of the anti-LRP6 binder 10SA7 HC to generate 1RC07-5: 10SA7 L->H. The LC of anti-LRP6E1E2 binder 1115.3 (as described in PCT Publication WO2009/064944) was fused to the N-terminus of the anti-FZD binder R2M3 HC with 5 or 10-mer linkers to generate 1115.3:5:R2M3 L->H or 1115.3:10:R2M3 L->H, respectively. These molecules also activated Wnt signaling (Fig. 15F and 15G).

Example 16

Characterization of R2M3-26 in the F(ab')2 Format

R2M3-26 IgG1 was digested by IDES (Promega, WI) at 37℃ for 2 hours. Vast majority of the digested product was R2M3-26F(ab')2 (Fig. 16A), some partially digested product with one Fab still attached to Fc (designated as R2M3-26F(ab')2-Fc here) was also detected, and no uncleaved R2M3-26 was detected. The cleaved product was purified by anti-Lambda resin to remove the Fc fragment, then a SEC polishing was followed to separate the R2M3-26F(ab')2 from R2M3-26F(ab')2-Fc. The SDS-PAGE gel of the final purified protein is shown in Fig.16B. The R2M3-26F(ab')2 activity was measured in STF assay in HEK293 cells. R2M3-26F(ab')2 was able to activate Wnt signaling (Fig. 16C).

Example 17

Characterization of Additional Wnt Surrogate Molecules

The FZD binders were fused to LRP binders. The LRP5 or 6 binders were Nabs (or VHHs) in this example and were fused to the N-terminus of FZD binders LC with a 5-amino acid linker (as represented in Fig. 17A). These proteins were purified by Protein A affinity column followed by a SEC step. The purified proteins were tested in Wnt dependent reporter assay in 293 cells (Fig. 17B, C, D, H), or 293 cells co-transfected with FZD4 expression construct (Fig. 17E, F), or 293 cells co-transfected with FZD9 expression construct (Fig. 17G) in the presence of 20nM R-spondin. These molecules activated Wnt signaling with varying levels of potency and efficacy.

Example 18

Characterization of 10SG11-1RC07 in the 2Fv-Ig Format

The molecule 10SG11-1RC07 consists of an N-terminal LRP binder (10SG11) and a Fzd binder (1RC07). The Fv of 10SG11 was fused to the N-terminus of 1RC07 with a 5-amino acid linker as depicted in Fig. 18A. 1RC07 was in the form of an IgG1 with the Fc mutations L234A/L235A/P329G. The protein was purified by a Protein A affinity column followed by an SEC step. The fusion protein was tested in Wnt dependent reporter assays in 293 cell lines and demonstrated the ability to activate Wnt signaling (Fig. 18B-C)

Example 19

In Vivo PK/PD Characterization of R2M3-26

Six-week old C57Bl/6J male mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were housed 3 per cage. All animal experimentation was in accordance with the criteria of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by the Surrozen Institutional Animal Care and Use Committee. Mice were acclimatized a minimum of two days prior to initiating experiments. Mice had unlimited access to purified, laboratory-grade acidified water and were fed *ad libitum* (2018 Teklad global 18% protein rodent diet) Mice were kept 12/12-hour light/dark cycle in a 30% to 70% humidity environment and room temperature ranging from 20°C to 26°C.

For the pharmacokinetic (PK) study (Fig. 20A), n=3 per group was used. Mice were dosed with R2M3-26 (with effectorless Fc mutations) at 1 mg/kg (10ml/kg in saline) either using intravenous (IV) or intraperitoneal (i.p.) injections. Mice were anesthetized with isoflurane and blood was removed from the retro-orbital plexus, tail vein or heart at 10 minutes, 30 minutes, 1, 4, 24, 72 or 144 hours after injection. Blood was allowed to coagulate at room temperature, followed by centrifugation for 7 minutes at 8,000 g. The serum was removed and stored at -20° C until the measurement of serum R2M3-26 concentrations by ELISA with Anti Human IgG Fc Fragment (Jackson Immuno Research Labs NC9747692).

For the pharmacodynamic (PD) study (Fig. 20B), n=6 per group was used. Mice were injected i.p. with R2M3-26 at the indicated doses (10ml/kg in saline). Control mice received saline only. Eight hours later, mice were anesthetized with isoflurane and the blood was collected by cardiac puncture. Blood was allowed to coagulate at room temperature, followed by centrifugation for 7 minutes at 10,000 g. The serum was removed and stored at -20°C until the measurement of serum R2M3-26 concentrations by ELISA.

A portion of the left liver lobe was snap-frozen in liquid nitrogen and stored at -80°C for RNA analysis. RNA was extracted from liver samples using the MagMAX™ mirVana™ Total RNA Isolation Kit (ThermoFisher, A27828). cDNA was produced using the high-Capacity cDNA Reverse Transcription Kit (ThermoFisher, 43-688-14). Axin2 mRNA expression was measured by using TaqMan® Fast Advanced Master Mix (ThermoFisher, 4444963) and the Mm00443610_m1 Axin2 Probe (Thermofisher, 4331182).

These studies showed that R2M3-26 was stable, highly bioavailable and active in vivo, as shown by the induction of Axin2 mRNA expression.

Example 20

In Vivo Bone Model and Characterization of AAV-Delivered Wnt Surrogates

In vivo experiments were conducted by infecting mice with an AAV vector that expressed Flag- and His-tagged 18R5-DKK1c protein (AAV-18R5- DKK1c-FlagHis). 18R5-DKK1c is a fusion protein containing the frizzled binding antibody, 18R5, in scFv format, fused to DKK1c, as described in PCT Publication WO2016/040895, e.g., Fig. 5. Control mice were treated with vehicle only, romosozumab, an AAV vector that expressed green fluorescent protein (GFP) (AAV-CAG-GFP), or an AAV vector that expressed a fusion protein comprising an anti-GFP scFv fused to a mutant DKK1c (AAV-ScFv (anti-GFP)-DKK1cF234K-Flag-His). 28 days after infection, animals were sacrificed and bone mineral density, bone volume and other characteristics were measured. As shown in Figs. 21A-21E, systemic expression of 18R5-DKK1c resulted in significantly increased bone mineral density as early as 14 days of 18R5-DKK1c systemic expression, as determined by dual X-ray absorptiometry (DEXA) scan. Systemic expression of 18R5-DKK1c increased bone mineral density (BMD) as measured by DEXA scan (FIG. 21A) and increased levels of the serum P1NP bone formation marker in naïve mice (FIGS. 21C). Serum levels of AAV-ScFv (anti-GFP)-DKK1cF234K and 18R5-

DKK1c were detected in the serum and found to be well above the in vitro determined EC50 (FIG. 21B). AAV-CAG-GFP and AAV-ScFv (anti-GFP)-DKK1cF234K were negative controls. Romosozumab was a positive control, and vehicle only was a negative control. 18R5-DKK1c also increase bone density in lumbar vertebra and whole body, as shown in FIGS. 20D and 20E, wherein * indicates P value <0.05 and** indicates P value <0.0001. 18R5-DKK1c expression through AAV also increased bone volume in tibia and femur and cortical thickness in femur mid-diaphysis at 28 days after treatment in naïve mice as measured by micro CT, as shown in Figs.22A-22D, wherein **** indicates P value <0.0001.

Systemic expression of 18R5-DKK1c resulted in significantly increased mineral apposition rate from baseline to single label in last 8 days, as shown in FIGS 23A and 23B.

Systemic expression of 18R5-DKK1c also resulted in increased osteoblast numbers and decreased osteoclast numbers, as shown in FIGS. 24A-24D.

18R5-DKK1c treatment increased bone stiffness and ultimate load to fracture in biomechanical testing, suggesting improved resistance to fracture, as shown in FIGS. 25A-25C.

These studies demonstrated that systemic expression of 18R5-DKK1c using AAV increased bone mineral density (BMD) as measured by DEXA, and also showed that 18R5-DKK1c increased bone volume as measured by micro CT as early as 14 days of treatment. 18R5-DKK1c also increased cortical thickness 28 days after treatment. Systemic expression of 18R5-DKK1c resulted in significantly increased mineral apposition rate, increased osteoblast numbers, and decreased osteoclast numbers. It also increased bone stiffness and ultimate load to fracture, suggesting improved resistance to fracture.

Example 21

In Vivo Bone Model and Characterization of Wnt Surrogates Produced as Recombinant Proteins

In vivo experiments were conducted by treating mice with recombinantly produced R2M3-26 protein at various dosages via i.p. injection. Control mice were treated with vehicle only (negative control), romosozumab (positive control), anti-Beta-Galactosidase (negative control), or IgG2-anti-GFP (negative control). Bone mineral density (BMD) measured by DEXA and bone volume measured by micro CT were monitored longitudinally at indicated time points. Four weeks after treatment, animals were sacrificed and bone characteristics were measured. For single injection of R2M3-26, the experimental data was monitored and is shown for two weeks after treatment.

Treatment with recombinant R2M3-26 induced rapid and sustained increase of bone mineral density (BMD) and bone volume in naive mice, as shown in FIGs. 26A-26D. Both bone volume and BMD increased rapidly, suggesting resistance to fracture.

The ovariectomy induced osteoporosis model is a well-established high-hurdle model for determining the ability of an anabolic therapy to overcome the bone loss associated with hormone ablation (Zhou, S. et. al., Journal of Cellular Biochemistry, PMID: 11455579). Treatment with recombinant R2M3-26 treatment reversed bone loss in an ovariectomy-induced osteoporosis mouse model, as shown in FIGS. 27A-27C. Increased cortical thickness was observed in the trabecular regions, suggesting increased compressive strength. R2M3-26 treatment increased femur mid-diaphysis cortical bone thickness after 42 days as measured by micro CT, as shown by FIG. 27D. BMD was also increased by R2M3-26 as measured by DEXA, shown in FIG. 27E.

A single injection of R2M3-26 was sufficient to induce rapid bone formation and bone volume within one week, as shown in FIGS. 28A-28C, wherein * indicates P value <0.05.

High dose treatment with R2M3-26 and 1R-C07-26 rapidly and significantly increased bone volume and bone mineral density, and improved biomechanical strength of the bone (ultimate load to failure and stiffness), as shown in FIGS. 29A-29D. 1R-C07-26 showed a robust and significant effect on bone accrual that persisted through 28 days. Both R2M3-26 and 1R-C07-26 significantly increased the resistance to fracture after 28 days of treatment by biomechanical testing.

High dose treatment with R2M3-26 and 1R-C07-3 rapidly and significantly increased bone volume, bone mineral density, and cortical thickness after only 14 days of treatment, as shown in FIG. 30A-E. 1R-C07-3 at 10 mpk appeared more effective at increasing bone mass than any other treatment tested in this preclinical model.

These studies demonstrate that recombinant protein treatment can induce rapid and sustained increase of bone mineral density and bone volume in naive mice and mouse osteoporosis model. Both bone volume and bone mineral density (BMD) increased rapidly, suggesting resistance to fracture. IgG2-anti-GFP is a negative control. Anti-Beta Galactosidase (anti-βgal) was a negative control.

An additional experiment was done to determine systemic skeletal effects of Wnt surrogate molecules in an ovariectomy-induced model of osteoporosis. C57BL/6 females, 4 weeks old at time of ovariectomy, (n=8/group) were compared to sham surgery operated as well as age-matched naïve mice. Animals were injected i.p. with recombinant Wnt surrogate molecules 7 months after surgery and when the onset of osteoporosis was confirmed. Experimental groups included R2M3-26, 1RC07-3, anti-Bgal (Ab control), and vehicle (PBS). Sub-cutaneous injection of romosozumab was done to another cohort of mice for comparison. Animal were treated twice weekly, and followed for 4 weeks.

As shown in FIG. 31, whole body bone mineral density (BMD) was measured weekly using dual-energy X-Ray absorptiometry (DEXA) and treatment with Wnt surrogate molecules can not only reverse but even

surpass the total BMD seen in naïve or non-surgical animals. After 4 weeks of treatment, animals were assessed for the vertebral resistance to compression fracture.

Treatment with Wnt surrogate molecules significantly increased the vertebral resistance to compression fracture, as shown by fracture analysis (FIG. 32). 1RC07-3 most robustly increases the Max Force required to compressively fracture the vertebra.

The Einhorn fracture model (Bonnarens F, Einhorn TA. J Orthop Res. 1984;2(1):97-101.PMID: 6491805) with delayed treatment with Wnt surrogate molecules was used to determine ability of this therapy to induce fracture healing. Delayed treatment with either 1RCO7-3 or R2M3-26 was tested to determine if either molecule was capable of contributing to increased fracture healing after a mid-traverse femoral fracture. C57BL/6 females, 16 weeks old at time of fracture (n=8/group) were used. The presence of a cartilaginous callus 2 weeks after fracture was confirmed in all animals before the start of treatment. With delayed treatment to allow for callus formation, a pure osteogenic signal could be elicited, with rapid mineralization of the already existing callus.

Animals were injected i.p. with recombinant Wnt surrogate molecules with the following experimental groups: R2M3-26, 1RCO7-3, anti-Bgal (Ab control), and vehicle (PBS). Sub-cutaneous injection of romosozumab was done to another cohort of mice for comparison. Animal were treated twice weekly, and followed for 6 weeks. Radiography was used to visualize changes in mineralization of the callus throughout the experiment (FIGs 33A and B). An increase in the mineralization and size of the resulting callus with Wnt surrogate treatment is apparent at both 1 and 6 weeks of treatment. One week was sufficient to induce rapid mineralization that is predictive of rapid bone fracture healing and resistance to fracture. 1RCO7-3 appeared to induce mineralization to a greater extent than R2M3-26. Radiographs taken after 6 weeks of treatment show the persistence of the

highly mineralized callus in the 1RCO7-3 group, while some of the fracture callus in the R2M3-26 group has diminished (FIGs 33B and D).

Whole body DEXA was measured throughout the experiment to examine bone mineral density in not only the fractured femur, but also the contralateral, non-fractured, femur where the expected increases in bone mineral density occurred after treatment (FIGs 33C and D). This provides reduced risk of secondary fracture in the already fractured limb and the appendicular skeleton. BMD of contralateral femur at 42 days are shown in Figure 33C.

Following 6-weeks of treatment after the confirmation of callus, we scanned the femurs with micro-computed tomography and determined multiple parameters which have been associated with an increased resistance to fracture after healing. Callus tissue volume, the bone volume within that callus, and especially bone mineral content are all significantly increased within the region of interest examined (Figure 33D). Qualitatively, the reconstructions show the prevalence of thick osteoid and mineral within the treated fracture. These parameters suggest a robust resistance to fracture and indicate that delayed treatment with Wnt surrogate molecules, after the spontaneous cartilaginous callus formation, can initiate a rapid and significant increase in bone formation.

In another experiment, the dosing schedule was tested to determine whether Wnt surrogate molecule therapy can induce a significant bone anabolic effect, how long that effect persists, and after washout, to determine how the bone responds to additional treatment. Systemic skeletal effects were compared with variable dosing of 1RCO7-3 on anabolic effect, washout, and redosing after establishing baseline. C57BL/6 females, 12 weeks old (n=8/group) were injected i.p. with recombinant Wnt surrogate molecules with the following experimental groups: two groups with 1RCO7-3, one with anti-βgal (Ab control), and one with vehicle (PBS) on day 0 of the experiment. Sub-cutaneous injection of romosozumab was done to another cohort of mice on day 0 for comparison (romosozumab is an anti-sclerostin

antibody (Saag et al., N Engl J Med. 2017 Oct 12;377(15):1417-1427; PMID:28892457) that may reverse the bone loss associated with osteoporosis). Animals in 1RC07-3 treated groups had significant and rapid induction of bone formation by 14 days (FIG 34). One group received a second injection at day 14 to determine if the bone anabolic effect could be further enhanced. Interestingly, regardless of treatment, all treatment effects were reversed and normalized after 35 days. A 2-week period was allowed to return to baseline levels prior to a second round of treatment. On day 49, a second round of treatment was done to all experimental groups. The Wnt surrogate treated animals responded rapidly, however not to the same magnitude as with the initial treatment (FIG. 34). For all groups, 5 weeks after the last injection, new bone formation ceased. This indicates that a single injection is capable of significantly increasing bone formation, however the anabolic effect is rapidly lost. This suggests that an anti-resorptive agent may be required in combination with Wnt surrogate therapy to maintain the anabolic effect.

The mechanism of action of romosozumab relies upon the stimulation of bone formation by removing an inhibitor (sclerostin) of endogenous Wnt signaling. An experiment was done treating animals with Wnt surrogate molecules plus romosozumab to determine if Wnt surrogate molecule treatment was capable of synergizing with romosozumab in a combination study.

C57BL/6 males, 10 weeks old (n=8/group) were injected i.p. with recombinant Wnt surrogate molecules in combination with romosozumab in the following experimental groups: 1RC07-3 (0.1 mpk), 1RCO7-3 (1 mpk), 1RCO7-3 (10 mpk), 1RCO7-3 (0.1 mpk) + romosozumab (25 mpk), 1RCO7-3 (1 mpk) + romosozumab (25 mpk), 1RCO7-3 (10 mpk) + romosozumab (25 mpk), romosozumab (25 mpk) alone, anti-Bgal (Ab control), and vehicle (PBS). Animals were treated twice weekly and followed for 3 weeks.

Whole body BMD was measured weekly and results are presented in FIG 35. Conclusions from this study are that endogenous

romosozumab can stimulate additional bone growth in the presence of high dose 1RC07-3. These data further suggest that peak anabolic action has not yet been reached with 10 mpk 1RCO7-3 treatments. These data also show that romosozumab can stimulate bone formation even in the presence of 1RCO7-3. Overall, This study shows that Wnt surrogate molecule treatment can synergize with romosozmab to enhance the bone anabolic effect after only 21 days of twice weekly treatment.

Changes in gene expression in whole bone were measured in a time course after Wnt surrogate molecule therapy in mice to assess how this therapy regulates expression of genetic markers related to proliferation and osteogenesis. C57BL/6 females, 13 weeks old (n=5/group) were injected i.p. with 1RCO7-3 or anti-Bgal (Ab control) once. Sub-cutaneous injection of romosozumab was done to another cohort of mice for comparison. Cohorts of animals were sacrificed at 8, 24, 48 and 120 hours after treatment, and tibia bones and serum were isolated and flash frozen for RNA extraction. ELISA was used to measure levels of therapeutic molecules in serum over the course of the experiment as described above (Figure 36).

For purification of RNA from bone, the excised tibia from freshly sacrificed animals were processed as follows: the ends of the tibia were clipped to expose marrow cavity and marrow cavity was flushed with ice cold saline through 30 gauge needle, Steps were taken to ensure all muscle tissue and cartilage has been removed; bone appeared completely white with no red marrow component residual. Tibias were placed in 1.5 mL Eppendorf tubes and flash frozen in liquid nitrogen. To lyse, a single tissue lyser bead was placed in the tube with bone and Trizol was added directly to frozen bone and bead. Tissue lyser at high speed was used to completely homogenize. Homogenates were then subject to chloroform extraction to separate the nucleic acid phase. Further isolation and purification was carried out using an RNeasy mini kit (Qiagen).

RNA Isolated from tibias was tested for relative transcript levels of Runt Related Transcription Factor 2 (RunX2), Collagen Type I Alpha 1

Chain (Col1A1), Dentin Matrix Acidic Phosphoprotein 1 (Dmp1), Alkaline phosphatase (Alp), Receptor activator of nuclear factor kappa-B ligand (RankL), Dickkopf WNT Signaling Pathway Inhibitor 1 (Dkk1), sclerostin (Sost), Cyclin D1 (Ccnd1), Axin2, and Ki67.

Table 4. Changes in gene expression in bone with Wnt surrogate molecule (1RC07-3) and romosozumab (Rxmab) Therapy in mice

gene	Time(hr)	Fold Induction +/- SEM relative to Bgal mean value		
		Bgal	Rzmab	1RC073
RunX2	24	1.0+/-0.3	8.8+/-4.4	9.6+/-3.6 **
	48	1.0+/-0.7	1.9+/-1.0	1.5+/-0.5
ColA1	24	1.0+/-0.2	3.9+/-2.0	3.0+/-1.0
	48	1.0+/-0.5	7.0+/-3.3	2.7+/-1.5
Dmp1	24	1.0+/-0.2	2.7+/-1.1	4.8+/-2.9
	48	1.0+/-0.6	1.6+/-0.7	3.8+/-1.8
Alp	24	1.0+/-0.4	18.3+/-11.7	16.5+/-6.4 *
	48	1.0+/-0.6	4.0+/-1.8	5.0+/-1.9
RankL	24	1.0+/-0.3	8.6+/-4.5	8.7+/-3.3
	48	1.0+/-0.7	1.5+/-0.6	4.3+/-1.4
Dkk1	24	1.0+/-0.2	10.5+/-5.1	17.9+/-6.0 **
	48	1.0+/-0.5	4.3+/-2.5	2.8+/-1.3
Sost	24	1.0+/-0.2	2.1+/-1.0	2.5+/-0.9
	48	1.0+/-0.6	5.1+/-2.3	9.0+/-4.2 **
Ccnd1	24	1.0+/-0.4	3.3+/-0.8	3.4+/-1.8
	48	1.0+/-0.6	2.5+/-1.2	2.4+/-1.2
Axin2	24	1.0+/-0.5	3.2+/-1.1	6.1+/-1.8 **
	48	1.0+/-0.8	3.6+/-2.3	4.6+/-1.6 *
Ki67	24	1.0+/- 0.3	7.7+/-4.3	10.0+/-3.2
	48	1.0 +/-0.5	1.0+/-0.5	25.5+/-12.2 ***

* p<0.05, ** p<0.005, 2-way ANOVA comparison with Bgal control

*** p<0.001, 2-way ANOVA comparison with both Bgal and romosozumab

In comparison to anti-sclerostin antibody (romosozumab) treatment, gene expression signatures over the time points were distinct with Wnt surrogate molecule therapy, with induction of more robust Axin2 and Ki67 expression than that caused by romosozumab treatment.

Example 22

In Vivo Liver Regeneration Model and Characterization of AAV-Delivered Wnt Surrogates

In vivo experiments were conducted by infecting approximately 8-weeks old C57BL/6J mice with an AAV vector that expressed Flag- and His-tagged 18R5-DKK1c protein (AAV-18R5-DKK1c-FlagHis). 18R5-DKK1c is a fusion protein containing the frizzled binding antibody, 18R5, in scFv format fused to DKK1c, as described in PCT Publication WO2016/040895, e.g., Fig. 5. Control mice were either injected sub-cutaneously with phosphate-buffered saline (PBS) only or romosozumab (10mg/kg), or injected intravenously (IV) with an AAV vector that expressed green fluorescent protein (GFP) (AAV-CAG- GFP), or an AAV vector that expressed a fusion protein comprising an anti-GFP scFv fused to a mutant DKK1c (AAV-ScFv (anti-GFP)-DKK1cF234K-Flag-His). 28 days after infection, animals were weighed and sacrificed. The liver was weighed, and the liver to body weight ratio calculated. The content of the small intestine and colon was removed by flushing with phosphate-buffered saline and gentle pressure to expel the content. The small intestine and colon were then weighed.

Systemic expression of 18R5-DKK1c-FlagHis resulted in a significant liver weight increase (FIG. 37A). Systemic expression of the negative controls, eGFP or anti-eGFP-Dkk1cF234K, did not affect the liver to body weight ratio. Administration of the romosozumab recombinant protein or vehicle control, did not affect the liver to body weight ratio.

None of the treatments affected the small intestine (FIG. 37B) or colon (FIG. 37C) to body weight ratio.

These studies showed that 18R5-DKK1c-FlagHis increases liver weight, but not that of the small or large intestines. This suggests that 18R5-DKK1c-FlagHis can promote liver regeneration.

Example 23

In Vivo Liver Regeneration Model and Characterization of Recombinantly Produced Wnt Surrogates

In vivo experiments were conducted by treating mice with recombinantly produced anti-eGFP, R2M3-26, 1R-C07-26, romosozumab or Rspo2 proteins at various dosages. The Rspo2 protein is a fusion protein between a short splice variant of the Rspo2 gene and a human Fc fragment.

In one study, mice were housed 4 per cage and n=8 per treatment group were used. Approximately 8-weeks old C57BL6/J mice were administered recombinant proteins, anti-eGFP (1 mg/kg), R2M3-26 (1 or 10 mg/kg) or 1R-C07-26 (1, 5 or 10 mg/kg), twice weekly intra-peritoneally (i.p.) for four weeks. In addition, groups of mice were administered romosozumab (30 mg/kg) or PBS vehicle control, subcutaneously.

Mice were weighed at the beginning and throughout the treatment. None of the treatment with recombinant proteins affected total body weight significantly (FIG. 38A). On day 28, the liver was weighed, and the liver to body weight ratio calculated (FIG. 38B). The highest dose of R2M3-26 (10 mg/kg) resulted in a significant increase in liver to body weight ratio. None of the other treatment affected the liver weight significantly.

The increase in liver weight in response to R2M3-26 suggest that this recombinant protein can promote liver regeneration.

In another study, mice were housed 5 per cage and n=10 per treatment group were used. Approximately 8-weeks old C57BL/6J mice received a single i.p. injection containing anti-eGFP (0.56 mg/kg), R2M3-26 (0.3 mg/kg) or Rspo2 (0.46 mg/kg) alone, or with a combination of R2M3-26 (0.1 mg/kg) and Rspo2 (0.46mg/kg).

24 or 48 hours after injection, mice were euthanized. A portion of the left liver lobe was snap-frozen in liquid nitrogen and stored at -80oC for RNA analysis. Cyclin D1 and Ki67 expression were measured by performing qPCR using the Mm00432359_m1 Ccnd1 probe and the Mm01278617_m1 Ki67 probe (Thermofisher, 4331182). An additional portion of the left liver lobe was fixed in formalin and embedded in paraffin for immunohistochemistry analysis. Sections were stained with the anti-proliferating cell nuclear antigen (PCNA) (Abcam, ab18197) or anti-phospho-histone H3 (pH3) rabbit antibodies (Abcam, ab47297). The number of positive nuclei were counted using the image processing software, Image J.

Rspo2 alone increased Ki67 (FIG. 39A) and CyclinD1 (FIG. 39B) mRNA expression. In combination with R2M3-26, Rspo2 increased Ki67 and CyclinD1 expression further than with Rspo2 alone at 24 and 48 hours after treatment with recombinant proteins. Rspo2 alone increased the number of PCNA (FIG. 39C) and pH3 (FIG. 39D) positive nuclei in liver sections. In combination with R2M3-26, Rspo2 increased the number of PCNA and pH3 positive nuclei further than with Rspo2 alone at 48 hours after treatment with recombinant proteins.

These studies show that the proliferation markers, Ki67 mRNA, CyclinD1 mRNA and PCNA-positive nuclei, and the pH3 mitotic marker are induced by the R2M3-26 and Rspo2 recombinant proteins and suggest that these recombinant proteins can promote liver regeneration.

Example 24

In Vivo Chronic Liver Injury Model and Characterization of AAV-Delivered Wnt Surrogates

Two in vivo experiments were conducted in two, thioacetamide (TAA)- and CCl4-induced, liver cirrhosis mouse models, thioacetamide (TAA)- and CCl4-induced, to test the effect of AAV vectors expressing 18R5-DKK1c-FlagHis or Rspo2 protein on chronic liver injury. TAA was added to the drinking water of 6 weeks old C57BL/6J mice at a concentration of 300 mg/L throughout

the TAA treatment duration. Mice were housed 5 per cage and groups of n=10 were used, except for the control groups without TAA treatment where n=5 per group was used.

In Study 1 (FIGS. 40A, 40C, 40E, 40G-H), mice with (n=10) or without (n=5) TAA treatment were weighed and sacrificed after 9 weeks of TAA addition into the drinking water, to measure baseline values. Livers were weighed and liver samples were collected for mRNA and histological analysis. TAA supplementation was maintained in the drinking water of the remaining mice and they were injected IV with AAV vectors that expressed an enhanced green fluorescent protein (eGFP) (3e10 genomic particles (GC)), 18R5-DKK1c- FlagHis (3e10 or 1e11 GC) or Rspo2 protein (1e11 GC) or a combination of 18R5-DKK1c-FlagHis (3e10 GC) and Rspo2 (1e11 GC). 5 age-matched naïve animals (no TAA) were kept as a negative control. Three weeks after AAV injection, all mice were weighed and euthanized. Livers were weighed and liver samples were collected for mRNA and histological analysis.

Treatment with 18R5Dkk1FH or Rspo2 resulted in a significant increase of liver weight (FIG. 40C) and liver to body weight ratio (FIG. 40E) in mice undergoing a continuous exposure to TAA. Treatment with a combination of 18R5Dkk1FH and Rspo2 resulted in a further increase in liver weight and liver to body weight ratio beyond that observed with either treatment alone. The combination 18R5Dkk1FH and Rspo2 treatment resulted in a decrease of the fibrosis marker Col1a1 mRNA expression (FIG. 40G). Histological liver sections were stained with Sirius red to visualize the accumulation of collagen in fibrotic area (FIG. 40H). Quantification of the percentage of red are, using Image J analysis software, showed a significant increase of fibrotic area in TAA-treated mice. A combination of 18R5Dkk1FH and Rspo2 resulted in a reversal of fibrotic area increase when compared to mice treated with the eGFP negative control. Treatment with Rspo2 alone also resulted in a significant but smaller reversal than the combination treatment.

In Study 2, (FIGS. 40B, 40D, 40F), mice were exposed to TAA-supplemented water for 11 weeks, and were returned to standard drinking

thereafter, two days prior to AAV treatment. At the start of AAV treatment, mice exposed to TAA (n=10) or not (n=5) were weighed and sacrificed to collect liver samples for baseline measurements. The remaining mice were injected with AAV vectors that expressed an enhanced green fluorescent protein (eGFP) (1.3e11 genomic particles (GC)), 18R5-DKK1c-FlagHis (3e10 or 1e11 GC) or Rspo2 protein (1e11 GC) or a combination of 18R5-DKK1c-FlagHis (3e10 GC) and Rspo2 (1e11 GC). 5 age-matched naïve animals (no TAA) were kept as a negative control. Three weeks after AAV injection, all mice were weighed and euthanized. Livers were weighed and liver samples were collected for mRNA and histological analysis.

A similar liver weight and liver to body weight increase was observed in mice treated with 18R5-Dkk1c-FlagHis and Rspo2 either alone or in combination than what was observed in Study 1 (FIGS. 40D, 40F).

These studies show that 18R5-Dkk1c-FlagHis and Rspo2 can increase liver weight in a TAA-induced liver cirrhosis model and reduce fibrosis markers. These results suggest that 18R5-Dkk1c-FlagHis and Rspo2 can promote liver tissue repair after chronic liver injury.

Example 25

In Vivo Chronic Liver Injury Models and Characterization of Recombinantly Produced Wnt Surrogates

In vivo experiments were conducted in thioacetamide (TAA)-induced and CCl4-induced mouse models of liver cirrhosis by treating mice with recombinantly produced anti-eGFP, R2M3-26 and Rspo2 proteins.

In the TAA-induced cirrhosis model, six weeks old male mice were exposed to TAA-supplemented drinking water (300 mg/L) for approximately 22 weeks (FIG. 41A). TAA exposure was removed two days prior to beginning of treatment with recombinant proteins and mice were provided with fresh drinking water. Mice were housed 5 per cage and n=10 per treatment group were used. In a mono treatment study (FIGS. 41B, 41D, 41F, 41H, 41J, 41L), mice were injected i.p. with anti-eGFP (1 mg/kg) or Rspo2 (1 mg/kg) twice weekly. In a

combination treatment study (FIGS. 41C, 41E, 41G, 41I, 41K, 41M), mice were injected i.p. with anti-eGFP (1.3mg/kg) or a combination of R2M3-26 (0.3 mg/kg) and Rspo2 (1 mg/kg) twice weekly. Mice were then weighed and sacrificed at day 3, 7 or 14 days after beginning of treatment. Groups of control mice without exposure to TAA (n=5 per group) were euthanized at day 0 and day 14 in both studies.

Treatment with Rspo2 protein alone or in combination with R2M3-26 resulted in an increase in liver to body weight ratio (FIGS. 41B and 41C) and a transient stimulation of the Wnt signaling pathway as shown by an increase in Axin2 expression (FIGS. 41D and 41E). Treatment with Rspo2 protein alone or in combination with R2M3-26 induced the following proliferation markers: cyclinD1 (FIGS. 41F, 41G) and Ki67 (FIGS. 41H and 41I) mRNA expression, PCNA (FIGS. 41J, 41K) and pH3 (FIGS. 41L, 41M) positive nuclei.

In an additional study, plasma was collected for prothrombin time measurement. Coagulation time is impaired in TAA-induced cirrhosis model as exemplified by an increase in pro-thrombin (PT) test to normal value ratio in mice exposed to TAA when compared to normal mice without TAA exposure (FIG. 41N). Treatment with Rspo2 (1 mg/kg) and R2M3-26 (0.3 mg/kg) reversed the prolongation in PT time as shown by the decrease in PT_{test}/PT_{normal} ratio at day 7 and day 14 after biweekly Rspo2 and R2M3-26 treatment.

These studies show that Rspo2 and R2M3-26 can stimulate liver cell proliferation and improve hepatocytes functional activity such as pro-thrombin time in a TAA-induced liver cirrhosis model. These results suggest that Rspo2 and R2M3-26 can promote liver tissue repair in chronic liver disease.

In the CCl4-induced cirrhosis model, six-weeks old C57BL/6J male mice were injected i.p. with 2 ml/kg CCl4 in mineral oil, twice weekly for 8 weeks (FIG. 42A). 3 days after the last CCl4 injection, mice were injected i.p. twice weekly with the following recombinant proteins: anti-β-galactosidase (10mg/kg), Rspo2 (1 or 10 mg/kg), or a combination of R2M3-26 (0.3mg/kg) and Rspo2 (1 mg/kg). Three additional control groups were included: one group injected with CCl4 but no proteins, one group injected with mineral oil, and one untreated age-

matched naïve group. n=8 were used for each group. After two weeks of treatment with recombinant proteins, mice were weighed and sacrificed. Plasma was collected for pro-thrombin time measurement. Livers were weighed and liver samples were collected for histological analysis.

Treatment with R2M3-26 and Rspo2 resulted in a significant increase in liver to body weight ratio when compared to treatment with anti-β-galactosidase negative control (FIG. 42B). Treatment with Rspo2 (10 mg/kg) or a combination of R2M3-26 and Rspo2 resulted in a significant decrease in prothrombin time (FIG. 42C). Treatment with Rspo2 (10 mg/kg) or a combination of R2M3-26 and Rspo2 resulted in a significant reversal in fibrotic area, induced by CCl4 (FIG. 42D).

This study showed that Rspo2 and R2M3-26 can induce an increase in liver weight, improve hepatocytes functional activity such as pro-thrombin time and reduce fibrosis markers in a CCl4-induced liver cirrhosis model. These results suggest that Rspo2 and R2M3-26 can promote liver tissue repair in chronic liver disease.

Example 26

In Vivo Acute Liver Injury Model and Characterization of Recombinantly Produced Wnt Surrogates

In vivo experiments were conducted using acetaminophen- induced mouse models of acute liver injury by treating mice with recombinantly produced anti-eGFP, R2M3-26 and Rspo2 proteins.

Eight-week old C57BL/6 male mice were housed 5 per cage. n=10 were used per group. Mice were fasted overnight for 12 hours. Acetaminophen (APAP) was administered i.p. at a sublethal dose (300 mg/kg).

In a first study, anti-eGFP (0.3 mpk) or R2M3-26 (0.3 mpk) were injected i.p. either immediately after or 3 or 6 hours after APAP injection (FIG.43). Serum samples were collected at 24 and 48 hours after APAP injection for ALT measurements. Mice were sacrificed 48 hours after APAP injection and liver samples were collected for mRNA analysis.

R2M3-26 treatment did not affect ALT level significantly (FIG. 43B). Treatment with R2M3-26 induced cyclinD1 (FIG. 43C) and Ki67 (FIG. 43D) mRNA significantly, beyond the level induced by APAP treatment alone.

In a second study, human Fc (0.46 mg/kg) or Rspo2 (0.46 mg/kg) were injected i.p. either immediately after or 3 or 6 hours after APAP injection (FIG. 44). Serum samples were collected at 24 and 48 hours after APAP injection. Liver samples were collected at 48 hours after APAP injection. Rspo2 treatment did not affect ALT serum level significantly (FIG. 44B). Treatment with Rspo2 induced cyclinD1 (FIG. 44C) and Ki67 (FIG. 43D) mRNA significantly, beyond the level induced by APAP treatment alone.

In a third study, anti-eGFP (0.56 mg/kg) or a combination of R2M3-26 (0.1 mg/kg) and Rspo2 (0.46 mg/kg) were injected i.p. 3 hours after APAP administration. Serum and liver samples were collected at 24, 36, 48 and 60 hours after APAP injection for ALT measurements and mRNA analysis.

R2M3-26 and Rspo2 combination treatment did not affect ALT level significantly (FIG. 45B). Treatment with R2M3-26 and Rspo2 induced cyclinD1 (FIG. 45C) and Ki67 (FIG. 45D) mRNA significantly, beyond the level induced by APAP treatment alone.

An additional study was performed to evaluate the effect of Rspo2 and R2M3-26 on the survival of mice treated with a dose of 600 mg/kg of acetaminophen (FIG. 46). Anti-eGFP (0.3 mg/kg), R2M3-26 (0.3 mg/kg), Rspo2 (0.46mg/kg) or a combination of R2M3-26 (0.1 mg/kg) and Rspo2 (0.46 mg/kg) were injected i.p. 3 hours after APAP administration. Mice were monitored several times daily over the next 96 hours. A consistent trend in improving survival was observed in groups treated with R2M3-26 (FIG. 46B), Rspo2 (FIG. 46C) or a combination of R2M3-26 and Rspo2 (FIG. 46D).

These studies show that Rspo2 and R2M3-26 can induce proliferation markers beyond that induced spontaneously in APAP-induced acute injury model. These results suggest that Rspo2 and R2M3-26 can enhance liver tissue repair after an acute liver injury.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments. These and other changes can be made to the embodiments in light of the above-detailed description.

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

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CLAIMS

What is claimed is:

1. A soluble, multivalent, multispecific Wnt surrogate molecule, wherein the Wnt surrogate molecule comprises: (i) one or more region that specifically binds to one or more Frizzled (Fzd) receptor (a Fzd binding region); and (ii) one or more region that specifically binds to a Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) and/or a Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (a LRP5/6 binding region).

2. The Wnt surrogate molecule of claim 1, comprising one or more Fzd binding regions and one or more LRP5/6 binding regions.

3. The Wnt surrogate molecule of claim 1, wherein the one or more Fzd binding regions comprise one or more antigen-binding fragments of an antibody.

4. The Wnt surrogate molecule of claim 3, wherein the one or more antigen-binding fragments are selected from the group consisting of: IgG, scFv, Fab, and VHH or single domain antibodies (sdAb).

5. The Wnt surrogate molecule of any of claims 3-4, wherein the one or more Fzd antigen-binding fragments comprise: (i) CDRH1, CDRH2 and CDRH3 sequences set forth for any of the antibodies of Tables 1A or 1B; and/or (ii) CDRL1, CDRL2 and CDRL3 sequences set forth for any of the antibodies of Tables 1A or 1B, or a variant of said Fzd binding region comprising one or more amino acid modifications, wherein said variant comprises less than 8 amino acid substitutions in said CDR sequences.

6. The Wnt surrogate molecule of any of claims 3-5, wherein the one or more Fzd binding regions comprise an amino acid sequence having at least 90% identity

to any of the sequences set forth in SEQ ID NOs:1-65 or 129-132, or an antigen-binding fragment thereof.

7. The Wnt surrogate molecule of any of claims 1-6, wherein the one or more Fzd binding regions bind to one or more of Frizzled 1 (Fzd1), Frizzled 2 (Fzd2), Frizzled 3 (Fzd3), Frizzled 4 (Fzd4), Frizzled 5 (Fzd5), Frizzled 6 (Fzd6), Frizzled 7 (Fzd7), Frizzle 8 (Fzd8), Frizzled 9 (Fzd9), and Frizzled 10 (Fzd10).

8. The Wnt surrogate molecule of claim 7, wherein the one or more Fzd binding region binds to two or more of Frizzled 1 (Fzd1), Frizzled 2 (Fzd2), Frizzled 3 (Fzd3), Frizzled 4 (Fzd4), Frizzled 5 (Fzd5), Frizzled 6 (Fzd6), Frizzled 7 (Fzd7), Frizzled 8 (Fzd8), Frizzled 9 (Fzd9), and Frizzled 10 (Fzd10).

9. The Wnt surrogate molecule of claim 8, wherein the one or more Fzd binding region binds to: (i) Fzd1, Fzd2, Fzd7 and Fzd9; (ii) Fzd1, Fzd2 and Fzd7; (iii) Fzd5 and Fzd8; (iv) Fzd5, Fzd7 and Fzd8; (v) Fzd1, Fzd4, Fzd5 and Fzd8; (vi) Fzd1, Fzd2, Fzd5, Fzd7 and Fzd8; (vii) Fzd4 and Fzd9; (viii) Fzd9 and Fzd10; (ix) Fzd5, Fzd8 and Fzd10; or (x) Fzd4, Fzd5 and Fzd8; Fzd1, Fzd5, Fzd7 and Fzd8.

10. The Wnt surrogate molecule of any of claims 1-9, wherein the one or more LRP5/6 binding regions comprise one or more antigen-binding fragments of an antibody.

11. The Wnt surrogate molecule of claim 10, wherein the one or more antigen-binding fragments are selected from the group consisting of: IgG, scFv, Fab, and VHH or sdAb.

12. The Wnt surrogate molecule of any of claims 1-11, wherein the one or more LRP5/6 binding regions or antigen-binding fragments comprise: (i) CDRH1, CDRH2 and CDRH3 sequences set forth for any of the antibodies of Table 2; and/or (ii) CDRL1, CDRL2 and CDRL3 sequences set forth for any of the antibodies of Table 2,

or a variant of said LRP5/6 binding region comprising one or more amino acid modifications, wherein said variant comprises less than 8 amino acid substitutions in said CDR sequences.

13. The Wnt surrogate molecule of any of claims 10-12, wherein the one or more LRP5/6 binding regions comprise an amino acid sequence having at least 90% identity to any of the sequences set forth in SEQ ID NOs:66-88 or 133, or an antigen-binding fragment thereof.

14. The Wnt surrogate of any of claims 1-13, wherein the Fzd binding region and the LRP5/6 binding region comprise the sequences set forth in Table 3 for any of the Wnt surrogates disclosed therein.

15. The Wnt surrogate molecule of any of claims 1-14, wherein the Fzd binding region comprises a Fab, and the LRP5/6 binding region comprises a VHH or sdAb or a scFv.

16. The Wnt surrogate molecule of claim 15, wherein the Fab is present within a full immunoglobulin (Ig), optionally an IgG, comprising a light chain and a heavy chain.

17. The Wnt surrogate molecule of claim 16, wherein the LRP5/6 binding region is fused to the N-terminus or the C-terminus of the heavy chain or is fused to the N-terminus or the C-terminus of the light chain.

18. The Wnt surrogate molecule of claim 17, wherein the LRP5/6 binding region is fused to the heavy chain or the light chain via one or more linker moiety.

19. The Wnt surrogate molecule of any of claims 1-14, wherein the Fzd binding region comprises a VHH or sdAb or scFv, and the LRP5/6 binding region comprises a Fab.

20. The Wnt surrogate molecule of claim 19, wherein the Fab is present within a full immunoglobulin (Ig), optionally an IgG, comprising a light chain and a heavy chain.

21. The Wnt surrogate molecule of claim 20, wherein the Fzd binding region is fused to the N-terminus or the C-terminus of the heavy chain.

22. The Wnt surrogate molecule of claim 20, wherein the Fzd binding region is fused to the N-terminus or the C-terminus of the light chain.

23. The Wnt surrogate molecule of claim 21 or claim 22, wherein the Fzd binding region is fused to the heavy chain or the light chain via one or more linker moiety.

24. The Wnt surrogate molecule of any of claims 1-14, wherein the Fzd binding region comprises a Fab or Fv, and the LRP5/6 binding region comprises a Fab or Fv.

25. The Wnt surrogate molecule of claim 24, wherein the Fab of the Fzd binding region or the Fab of the LRP5/6 binding region is present within a full immunoglobulin (Ig), optionally an IgG, comprising a light chain and a heavy chain.

26. The Wnt surrogate molecule of claim 25, wherein only one of the Fab of the Fzd binding region or the Fab of the LRPp5/6 binding region is present within the full immunoglobulin (Ig).

27. The Wnt surrogate molecule of claim 26, wherein the Fab of the Fzd binding region is present within the full Ig.

28. The Wnt surrogate molecule of claim 27, wherein the Fab or Fv of the LRP5/6 binding region is fused to the N-terminus of the Ig, optionally the N-terminus of the heavy chain of the full Ig or the N-terminus of the light chain of the full Ig.

29. The Wnt surrogate molecule of claim 27, wherein the Fab of the LRP5/6 binding region is fused to the C-terminus of the Ig, optionally the C-terminus of the heavy chain of the full Ig or the C-terminus of the light chain of the full Ig.

30. The Wnt surrogate molecule of claim 27, wherein the variable light chain region of the LRP5/6 binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig.

31. The Wnt surrogate molecule of claim 27, wherein the variable light chain region of the LRP5/6 binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the LRP5/6 binding Fab is fused to the N-terminus of the variable light chain region of the full IgG.

32. The Wnt surrogate molecule of claim 27, wherein the variable light chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable light chain region of the full IgG.

33. The Wnt surrogate molecule of claim 27, wherein the variable heavy chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable light chain region of the LRP5/6 binding Fv is fused to the N-terminus of the variable light chain region of the full IgG.

34. The Wnt surrogate molecule of claim 26, wherein the Fab of the LRP5/6 binding region is present within the full Ig.

35. The Wnt surrogate molecule of claim 34, wherein the Fab or Fv of the Fzd binding region is fused to the N-terminus of the Ig, optionally the N-terminus of the heavy chain of the full Ig or the N-terminus of the light chain of the full Ig.

36. The Wnt surrogate molecule of claim 34, wherein the Fab or Fv of the Fzd binding region is fused to the C-terminus of the Ig, optionally the C-terminus of the heavy chain of the full Ig or the C-terminus of the light chain of the full Ig.

37. The Wnt surrogate molecule of claim 34, wherein the variable light chain region of the Fzd binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig.

38. The Wnt surrogate molecule of claim 34, wherein the variable light chain region of the Fzd binding Fab is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the Fzd binding Fab is fused to the N-terminus of the variable light chain region of the full IgG.

39. The Wnt surrogate molecule of claim 34 or claim 35, wherein the variable light chain region of the Fzd binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable heavy chain region of the Fzd binding Fv is fused to the N-terminus of the variable light chain region of the full IgG.

40. The Wnt surrogate molecule of claim 34 or claim 35, wherein the variable heavy chain region of the Fzd binding Fv is fused to the N-terminus of the variable heavy chain region of the full Ig, and the variable light chain region of the Fzd binding Fv is fused to the N-terminus of the variable light chain region of the full IgG.

41. The Wnt surrogate molecule of any of claims 1-14, wherein the Fzd binding region comprises a VHH or sdAb or scFv, and the LRP5/6 binding region comprises a VHH or sdAb or scFv.

42. The Wnt surrogate molecule of claim 41, wherein the Fzd binding region is fused to the Lrp5/6 binding region, and wherein the Fzd binding region or the LRP5/6 binding region is fused to an Fc region.

43. The Wnt surrogate molecule of claim 41, wherein the Fzd binding region is fused to the N-terminus of an Fc region, and wherein the LRP5/6 binding region is fused to the C-terminus of an Fc region.

44. The Wnt surrogate molecule of claim 41, wherein the Fzd binding region is fused to the C-terminus of an Fc region, and wherein the LRP5/6 binding region is fused to the N-terminus of an Fc region.

45. The Wnt surrogate molecule of any of claims 1-14, wherein the surrogate molecule has a structure set forth in Figs. 1A-D, 10A, 15A., 16A or 18A.

46. The Wnt surrogate molecule of any of claims 1-45, wherein the one or more antigen-binding fragment thereof, is humanized.

47. The Wnt surrogate molecule of any of claims 1-46, which binds to the one or more Fzd receptor with a KD of 50 μM or lower.

48. The Wnt surrogate molecule of any of claims 1-47, which binds to the one or more LRP5/6 receptor with a KD of 50 μM or lower.

49. The Wnt surrogate molecule of any of claims 1-48, which modulates a Wnt signaling pathway in a cell, optionally wherein the cell is a mammalian cell.

50. The Wnt surrogate of claim 49, wherein the Wnt signaling pathway is a canonical Wnt signaling pathway or a non-canonical Wnt signaling pathway.

51. The Wnt surrogate molecule of claim 49 or claim 50, which increases signaling via the Wnt signaling pathway in the cell.

52. An isolated polynucleotide encoding a polypeptide sequence comprising one or more of the Fzd binding regions and/or one or more of the LRP5/6 binding regions of a Wnt surrogate molecule of any of claims 1-51.

53. An expression vector comprising the isolated polynucleotide of claim 52.

54. An isolated host cell comprising the expression vector of claim 53.

55. A pharmaceutical composition comprising a physiologically acceptable excipient, diluent, or carrier, and a therapeutically effective amount of the Wnt surrogate molecule according to any of claims 1-51, the polynucleotide of claim 52, the expression cell of claim 53, or the host cell of claim 54.

56. A method for agonizing a Wnt signaling pathway in a cell, comprising contacting the cell the Wnt surrogate molecule according to any of claims 1-51, the polynucleotide of claim 52, the expression cell of claim 53, or the host cell of claim 54, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

57. A method for treating a subject having a disease or disorder associated with reduced Wnt signaling, comprising administering to the subject an effective amount of the Wnt surrogate molecule according to any of claims 1-51, the polynucleotide of claim 52, the expression cell of claim 53, the host cell of claim 54, or the pharmaceutical composition of claim 55, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

58. The method of claim 57, wherein the disease or disorder is a bone disease or disorder.

59. The method of claim 58, wherein the Wnt surrogate molecule binds Fzd1, Fzd2, and FZD7, and binds LRP5 and/or LRP6.

60. The method of claim 59, wherein the Wnt surrogate molecule also binds Fzd5 and Fzd8.

61. The method of claim 57, wherein the disease or disorder is selected from the group consisting of: bone fractures, stress fractures, vertebral compression fractures, osteoporosis, osteoporotic fractures, non-union fractures, delayed union fractures, spinal fusion, pre-operative optimization for spine surgeries, osteonecrosis, osseointegration of implants or orthopedic devices, osteogenesis imperfecta, bone grafts, tendon repair, tendon-bone integration, tooth growth and regenerationmaxillofacial surgery, dental implantation, periodontal diseases, maxillofacial reconstruction, osteonecrosis of the jaw, hip or femoral head, avascular necrosis, alopecia, hearing loss, vestibular hypofunction, macular degeneration, age-related macular degeneration (AMD), vitreoretinopathy, retinopathy, diabetic retinopathy, diseases of retinal degeneration, Fuchs' dystrophy, cornea diseases, stroke, traumatic brain injury, Alzheimer's disease, multiple sclerosis, muscular dystrophy, muscle atrophy caused by sarcopenia or chachexia, diseases affecting blood brain barrier (BBB) integrity, spinal cord injuries, spinal cord diseases, oral mucositis, short bowel syndrome, inflammatory bowel diseases (IBD), metabolic syndrome, diabetes, dyslipidemia pancreatitis, exocrine pancreatic insufficiency, wound healing, diabetic foot ulcers, pressure sores, venous leg ulcers, epidermolysis bullosa, dermal hypoplasia, myocardial infarction, coronary artery disease, heart failure, hematopoietic cell disorders, immunodeficiencies, graft versus host diseases, acute kidney injuries, chronic kidney diseases, chronic obstructive pulmonary diseases (COPD), idiopathic pulmonary fibrosis, acute liver failure of all causes, acute liver failure drug-induced, alcoholic liver diseases including alcoholic hepatitis, chronic liver failure of all causes, cirrhosis, liver fibrosis of all causes, portal hypertension, chronic liver insufficiency of all causes, nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD) (fatty liver), alcoholic hepatitis, hepatitis C virus-induced liver diseases (HCV), hepatitis B virus-induced liver diseases (HBV), other viral hepatitis (e.g., hepatitis A virus-induced liver diseases (HAV) and hepatitis D virus-induced liver diseases (HDV)), primary biliary cirrhosis,

autoimmune hepatitis, livery surgery, liver injury, liver transplantation, “small for size” syndrome in liver surgery and transplantation, congenital liver disease and disorders, any other liver disorder or detect resulting from genetic diseases, degeneration, aging, drugs, or injuries.

62. A method for increasing bone mineral density, increasing bone volume, increasing bone cortical thickness, increasing bone mineral apposition rate, increasing bone stiffness, increasing bone biomechanical strength, increasing resistance to bone fracture, or decreasing bone loss associated with osteoporosis, comprising providing to a subject an effective amount of the pharmaceutical composition of claim 55, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

63. The method of claim 62, wherein the Wnt surrogate molecule binds Fzd1, Fzd2, and FZD7, and binds LRP5 and/or LRP6.

64. The method of claim 63, wherein the Wnt surrogate molecule also binds Fzd5 and Fzd8.

65. A method for increasing liver to body weight ratio, promoting liver regeneration, increasing liver cell proliferation or mitosis, decreasing liver fibrosis, optionally following a chronic liver injury, increasing hepatocyte function, or decreasing coagulation time in liver, comprising providing to a subject an effective amount of the pharmaceutical composition of claim 55, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

66. The method of any of claims 57-65, further comprising providing to the subject an antiresorptive agent.

67. The method of claim 66 for the treatment of osteoporosis, optionally post-menopausal osteoporosis.

68. A method for inhibiting or reducing bone resorption in a subject in need thereof, comprising providing to the subject an effective amount of the Wnt surrogate molecule according to any of claims 1-51, the polynucleotide of claim 52, the expression cell of claim 53, the host cell of claim 54, or the pharmaceutical composition of claim 55, wherein the Wnt surrogate molecule is an agonist of a Wnt signaling pathway.

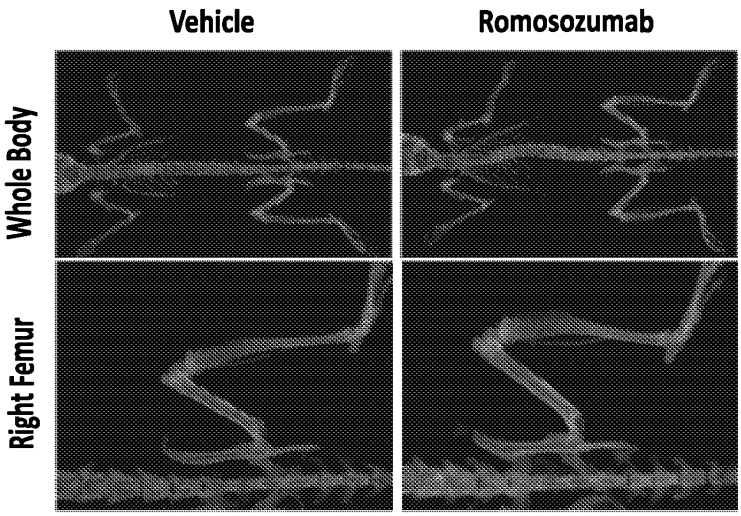
69. The method of claim 68, further comprising providing to the subject an antiresorptive agent.

70. The method of claim 68 or claim 69, wherein the subject has been diagnosed with or is at risk for osteoporosis, optionally postmenopausal osteoporosis.

ABSTRACT OF THE DISCLOSURE

The present invention provides Wnt pathway agonists and related compositions, which may be used in any of a variety of therapeutic methods for the treatment of diseases.

A.


Vehicle
Romosozumab
Whole Body
Right Femur

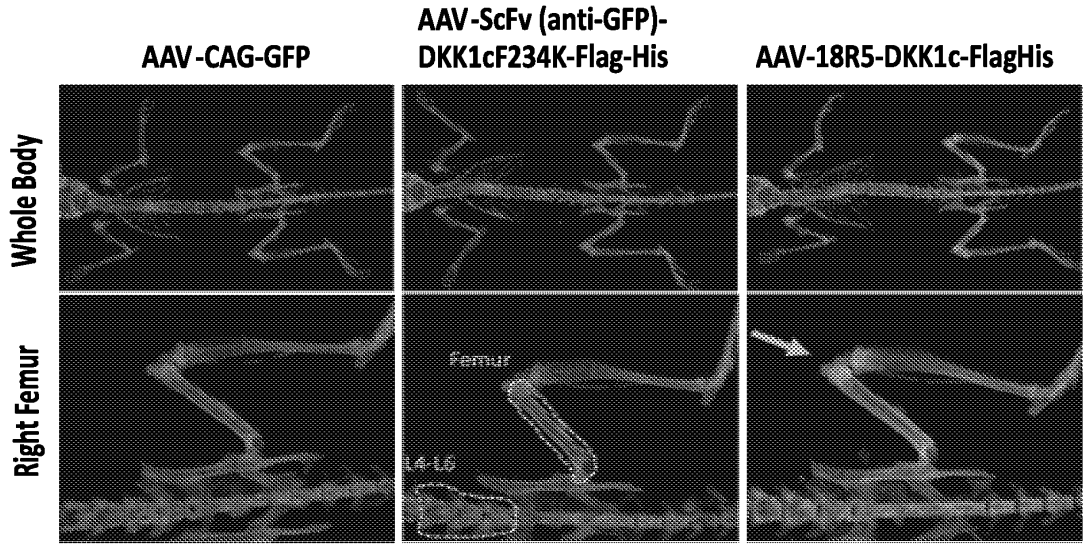

AAV-CAG-GFP
AAV-ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV-18R5-DKK1c-FlagHis
Whole Body
Right Femur
Femur
L4-L6

FIG. 21A

B.

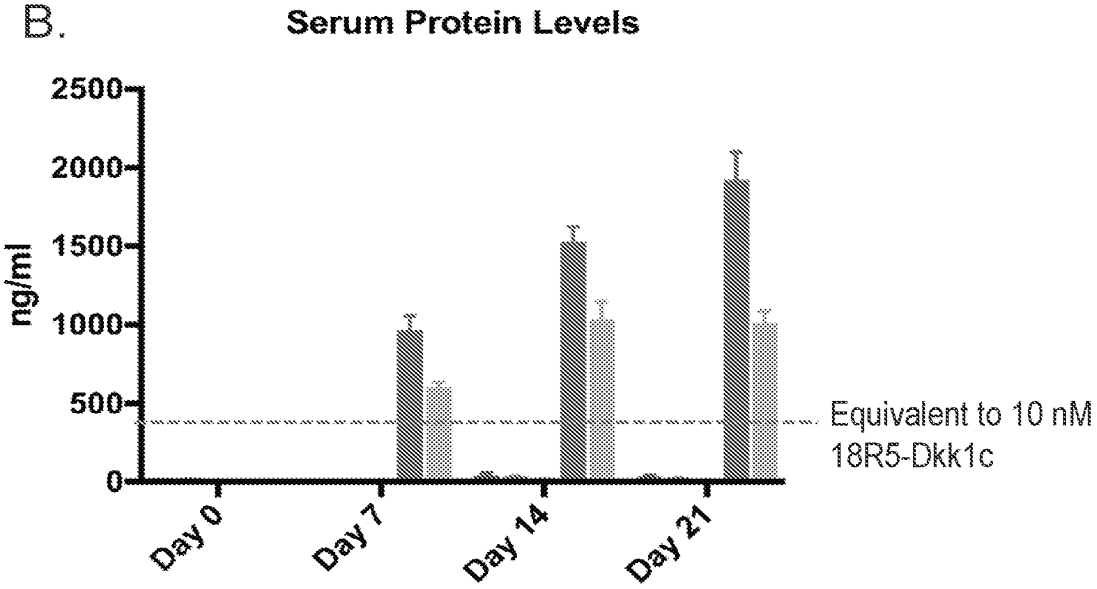


FIG. 21B

C.

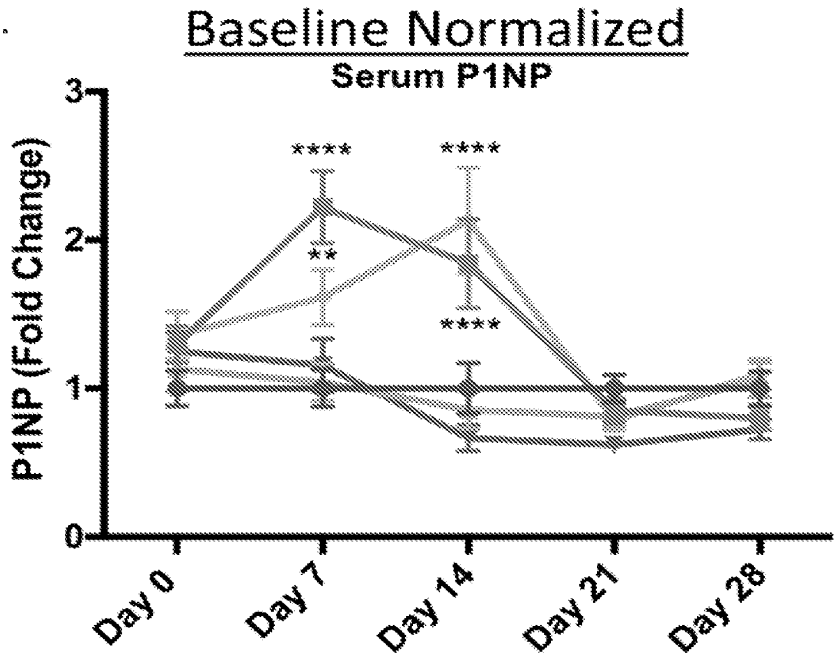


FIG. 21C

D.

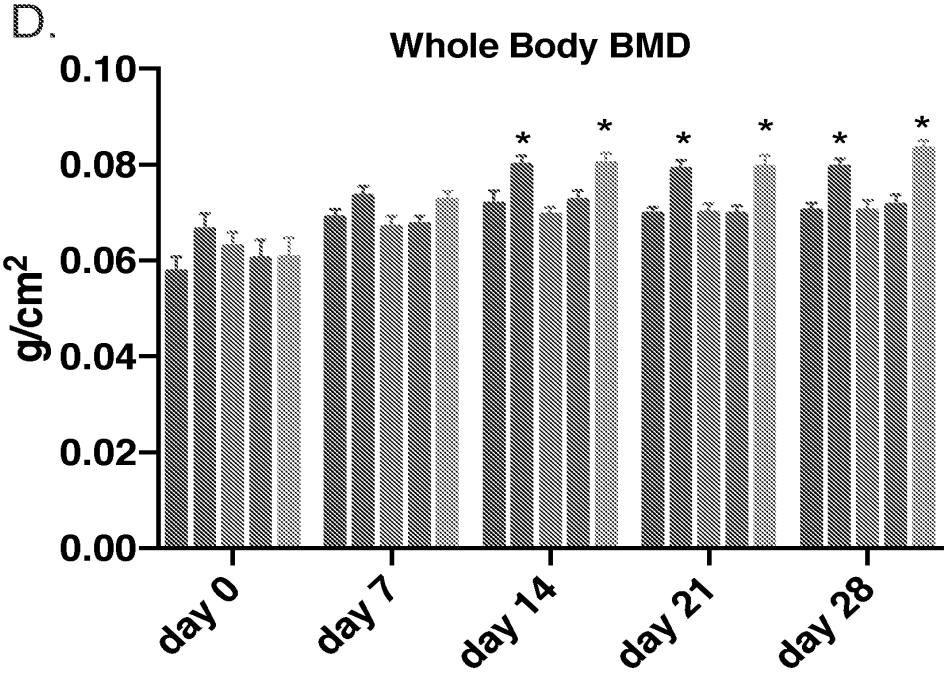
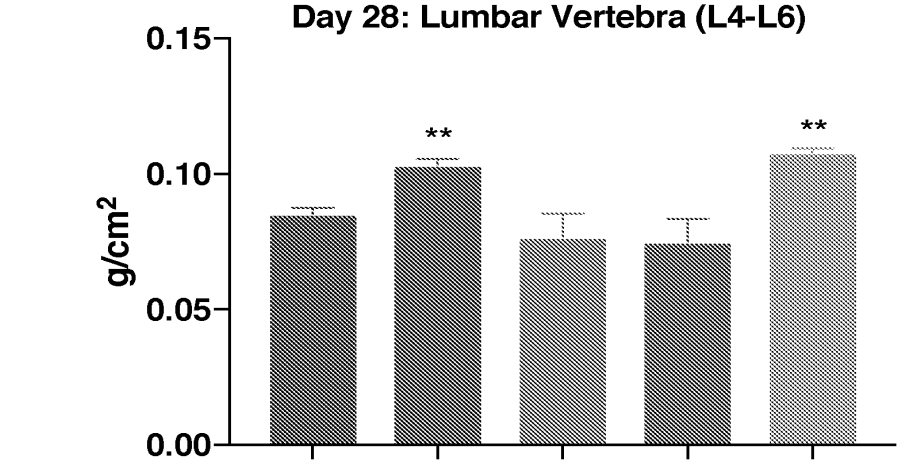


FIG. 21D

E.



Vehicle
Romosozumab
AAV -CAG-GFP
AAV -ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV -18R5-DKK1c-FlagHis

** P value <0.05
**** P value <0.000.1

FIG. 21E

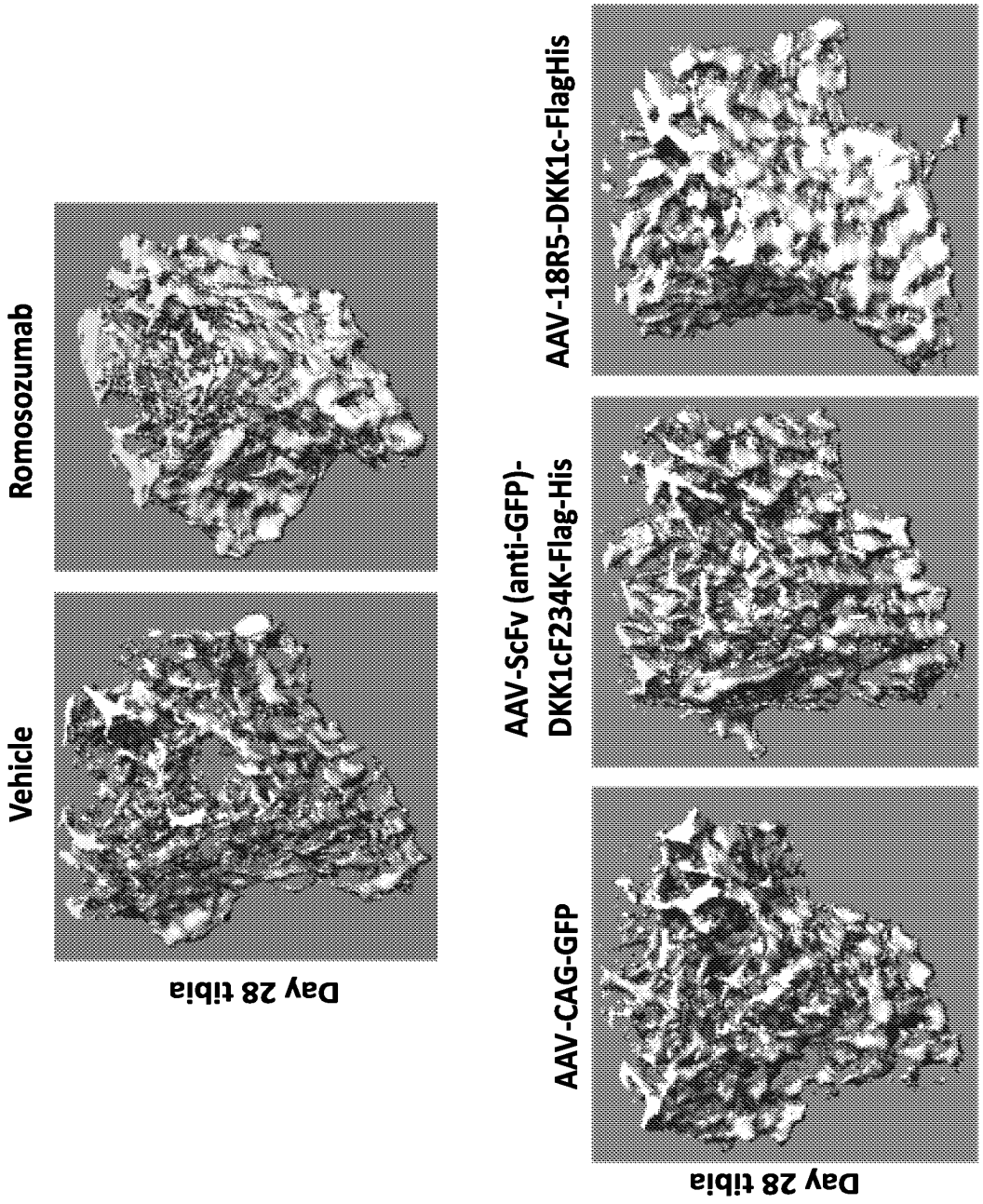

Vehicle
Romosozumab
Day 28 tibia
AAV-CAG-GFP
AAV-ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV-18R5-DKK1c-FlagHis
Day 28 tibia

FIG. 22A

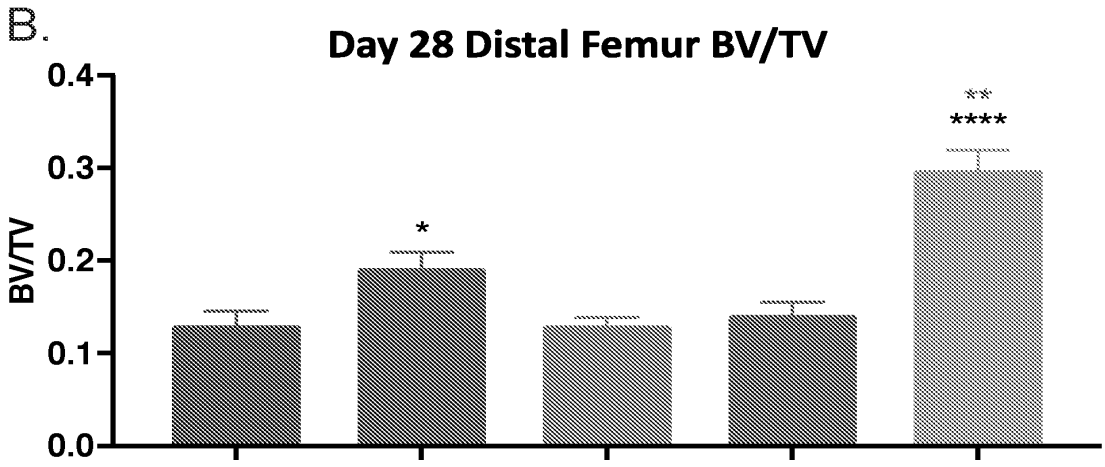


FIG. 22B

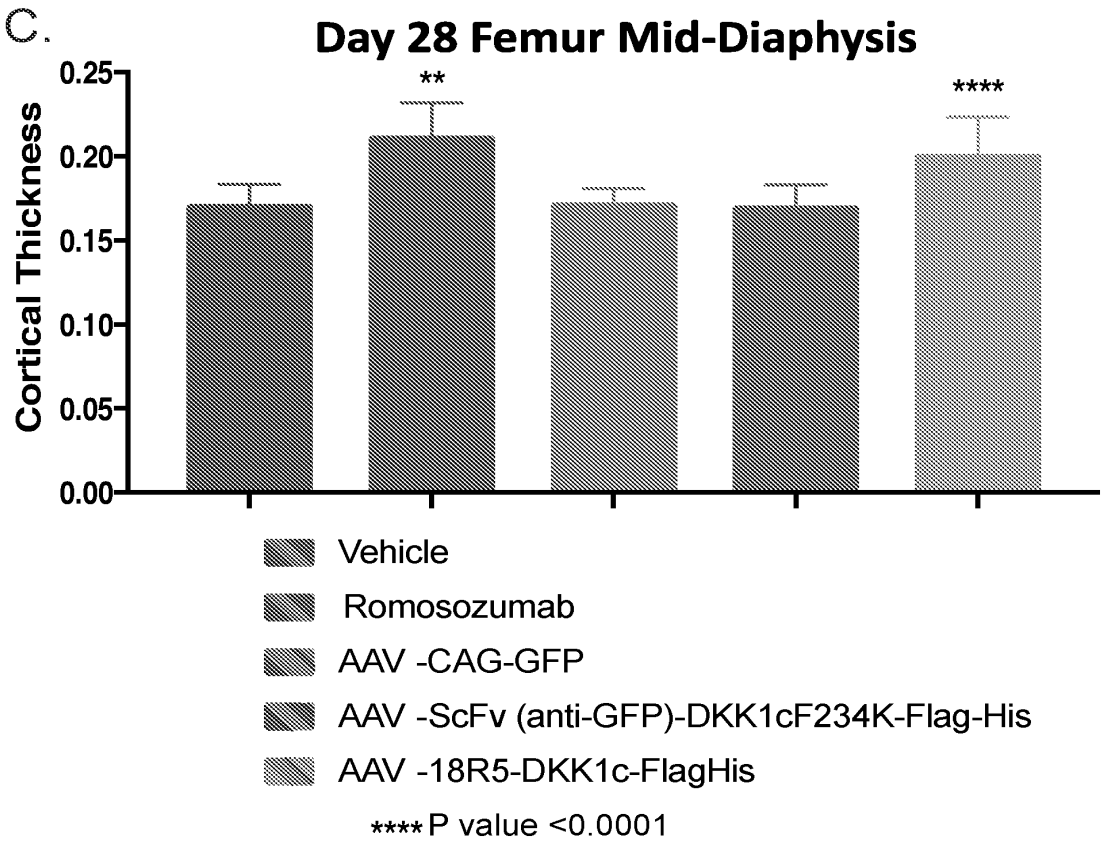


FIG. 22C

D.

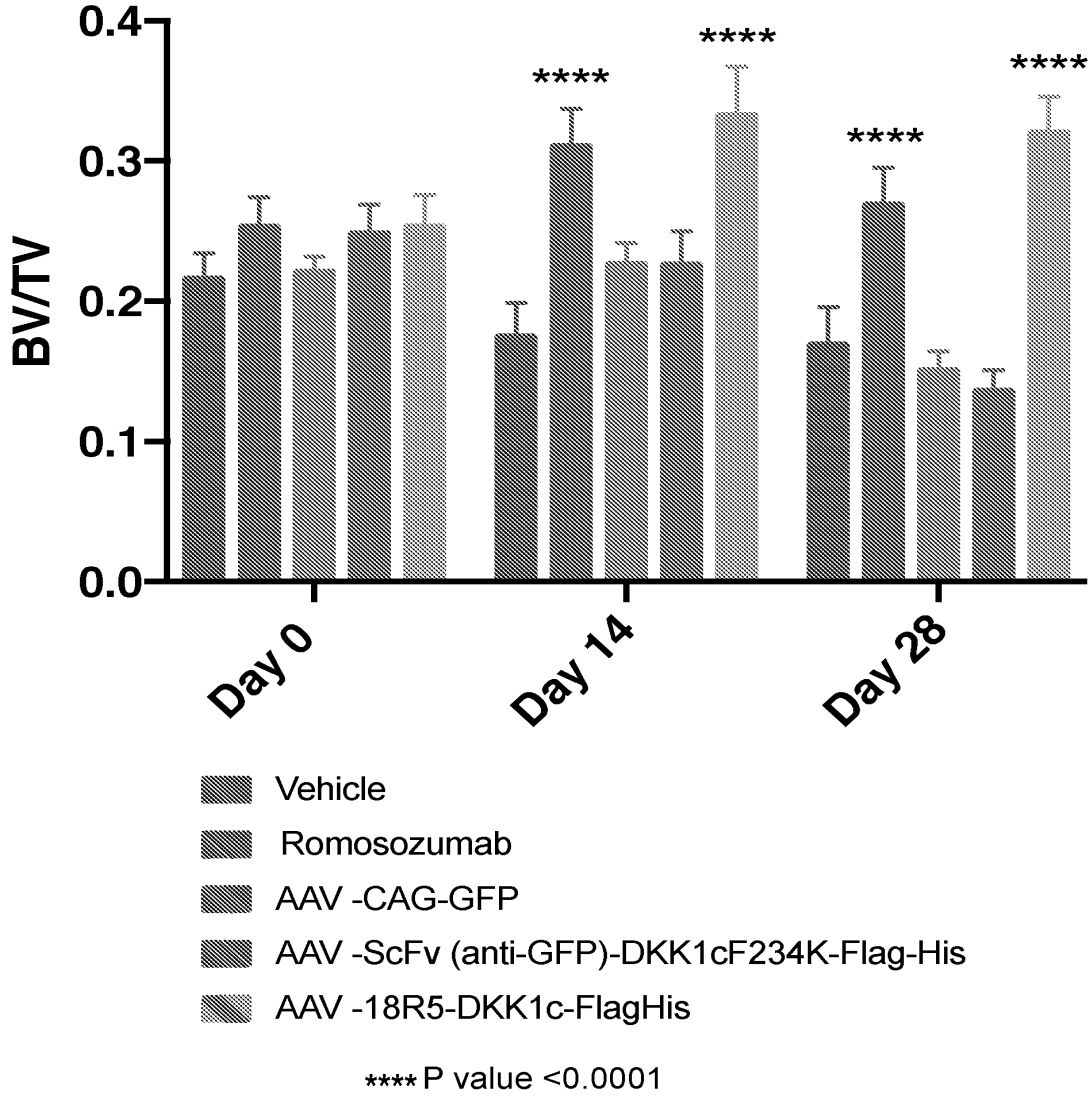

0.4
0.3
0.2
0.1
0.0
BV/TV
****
****
****
****
Day 0
Day 14
Day 28
Vehicle
Romosozumab
AAV -CAG-GFP
AAV -ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV -18R5-DKK1c-FlagHis
**** P value <0.0001

FIG. 22D

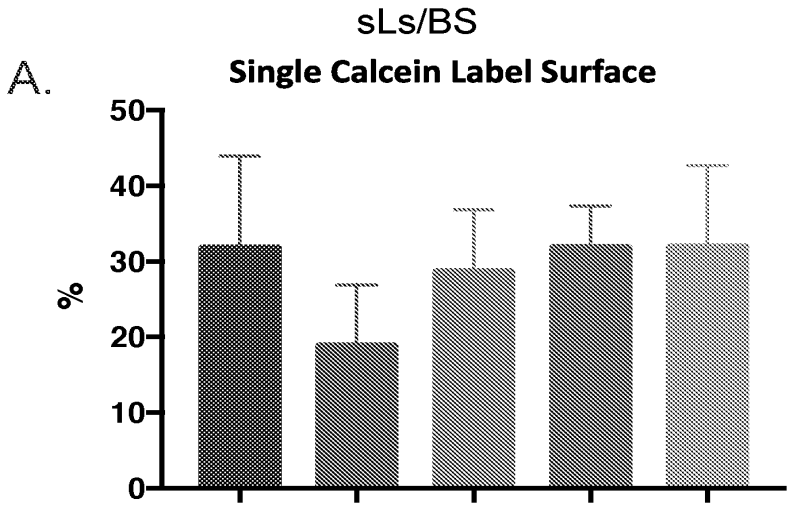


FIG. 23A

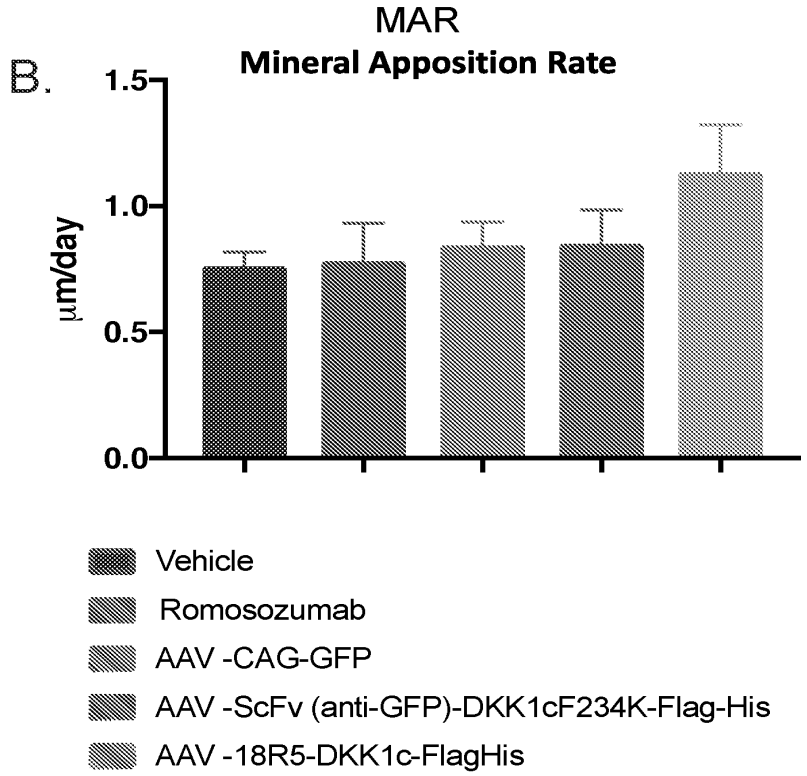


FIG. 23B

FIG. 24A-C

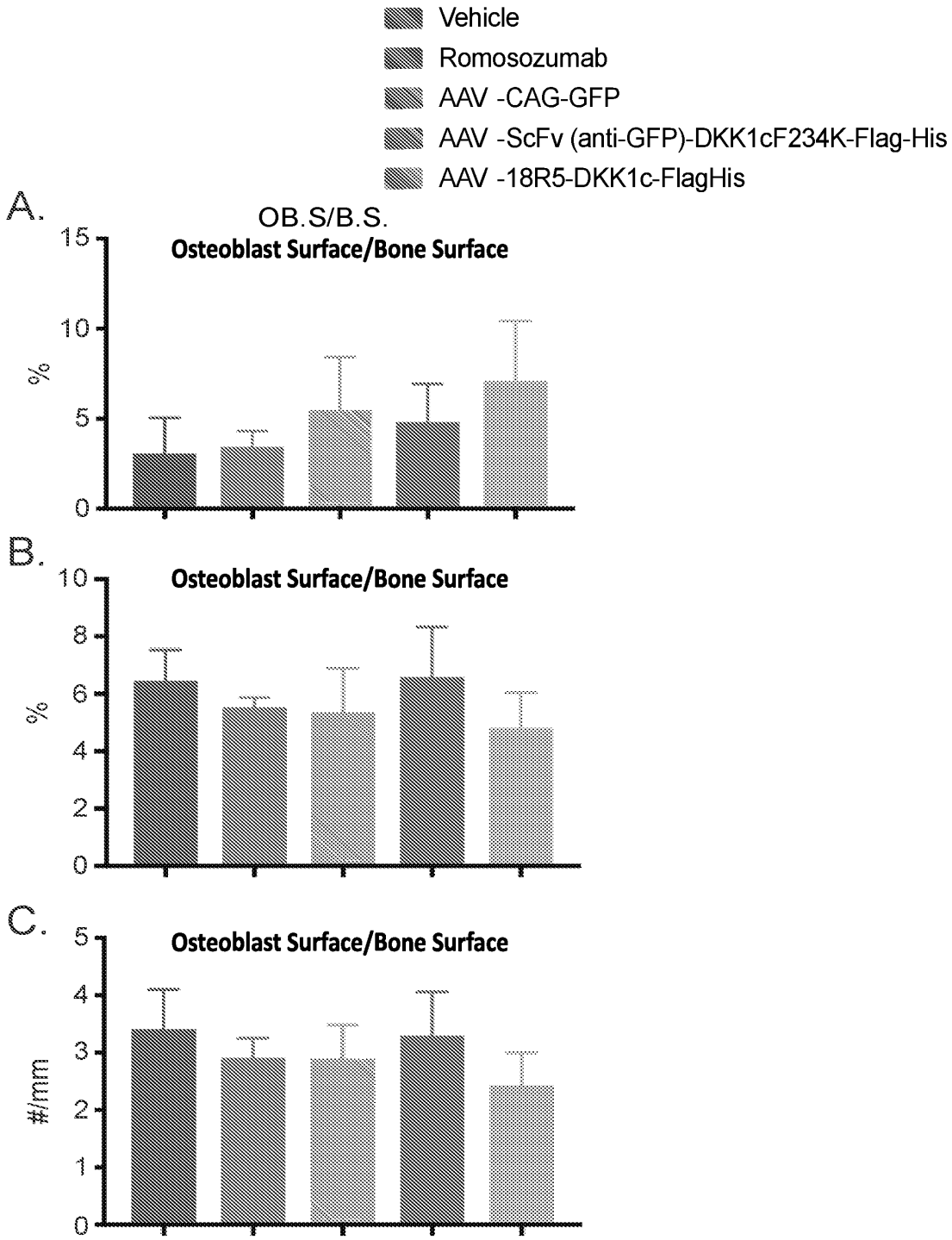

Vehicle
Romosozumab
AAV -CAG-GFP
AAV -ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV -18R5-DKK1c-FlagHis
A.
OB.S/B.S.
Osteoblast Surface/Bone Surface
%
15
10
5
0
B.
Osteoblast Surface/Bone Surface
%
10
8
6
4
2
0
C.
Osteoblast Surface/Bone Surface
#/mm
5
4
3
2
1
0

FIG. 24D

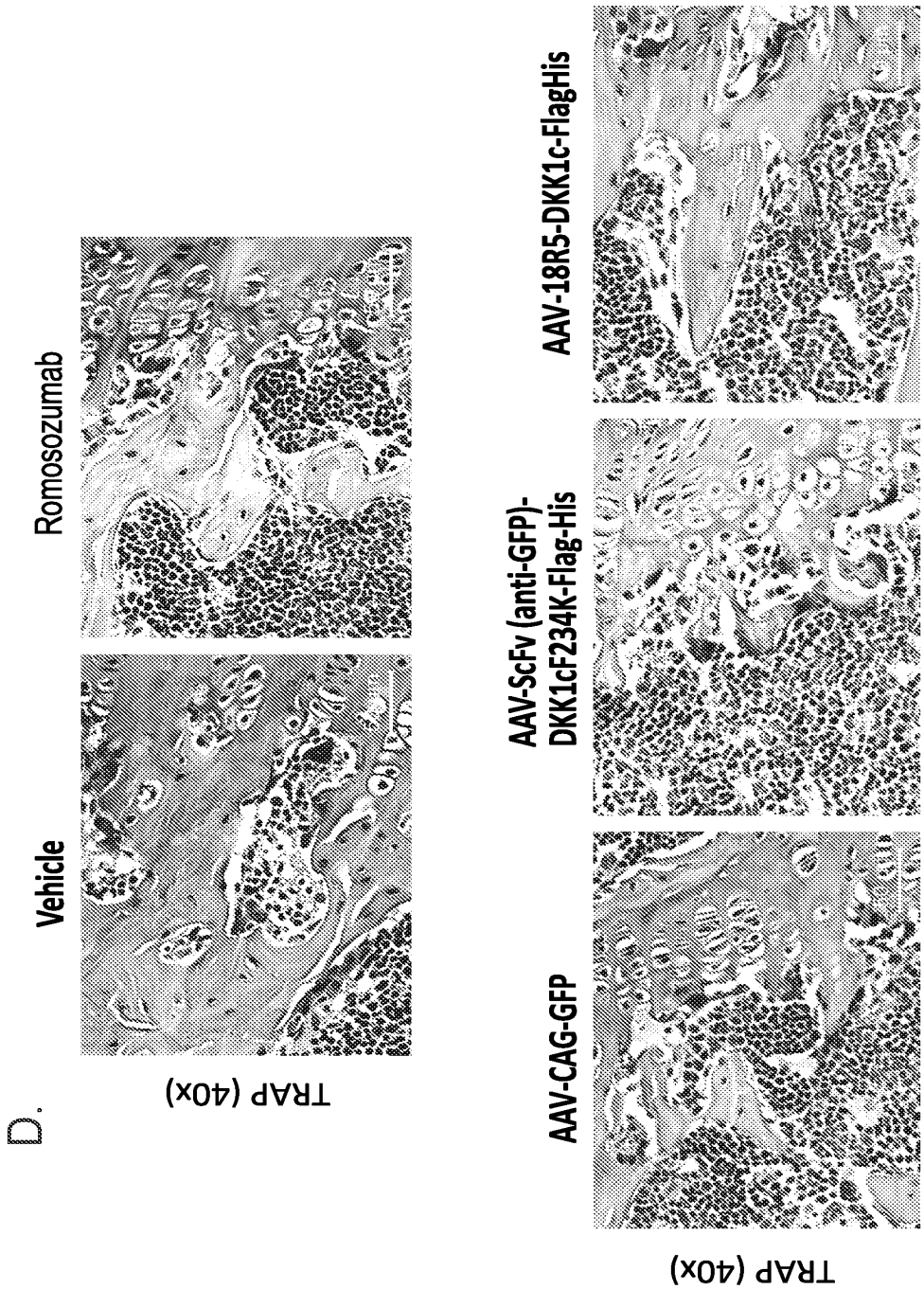

D.
Vehicle
Romosozumab
TRAP (40x)
AAV-CAG-GFP
AAV-ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV-18R5-DKK1c-FlagHis
TRAP (40x)

FIG. 25A

A.

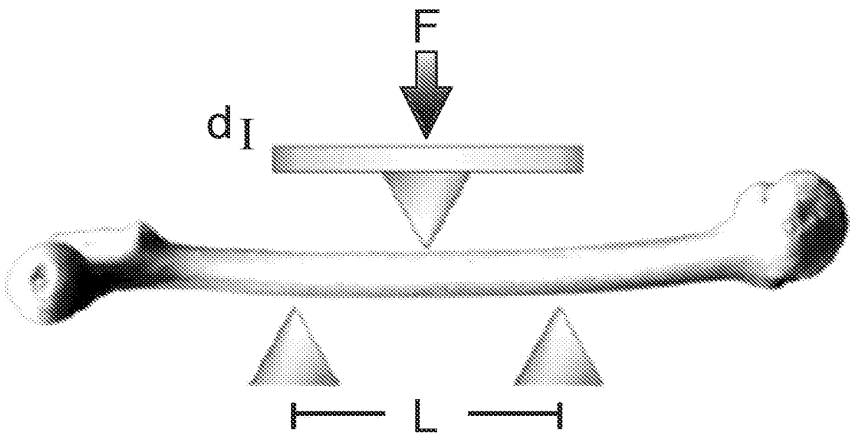
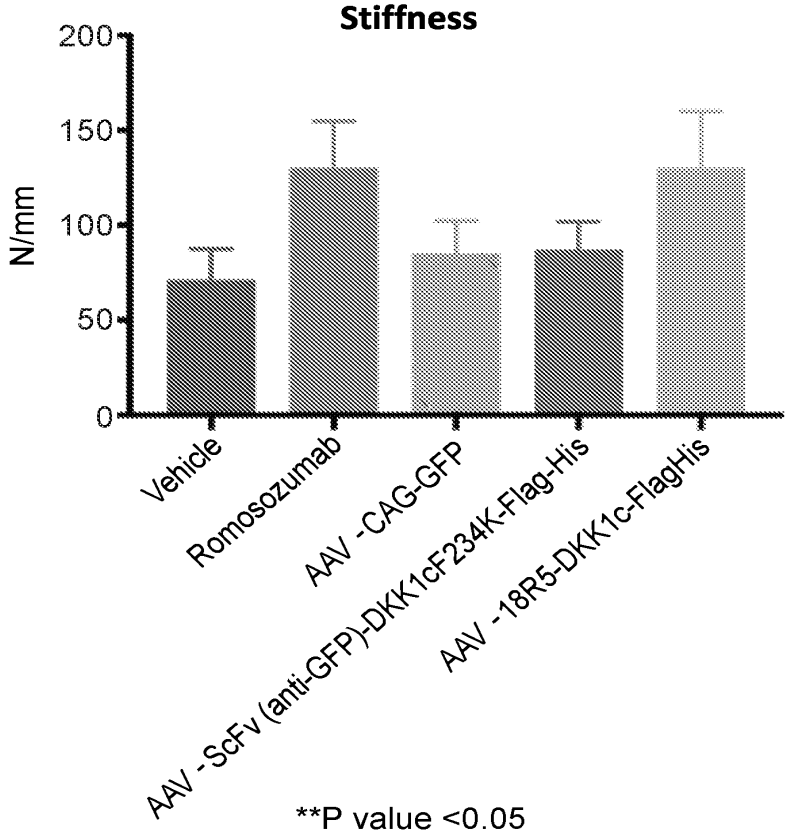


FIG. 25B

B.



**P value <0.05

FIG. 25C

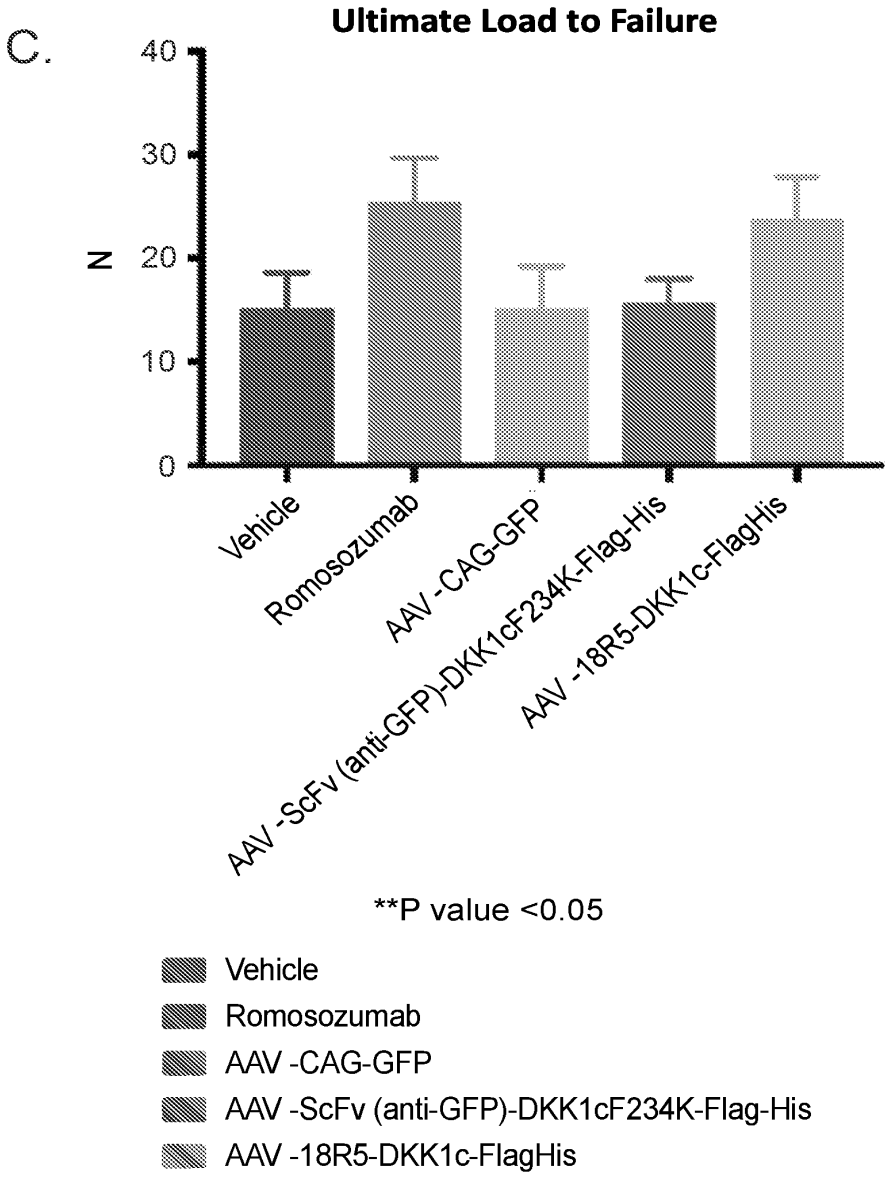

C.
Ultimate Load to Failure
N
40
30
20
10
0
Vehicle
Romosozumab
AAV -CAG-GFP
AAV -ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV -18R5-DKK1c-FlagHis
**P value <0.05
Vehicle
Romosozumab
AAV -CAG-GFP
AAV -ScFv (anti-GFP)-DKK1cF234K-Flag-His
AAV -18R5-DKK1c-FlagHis

FIG. 26A

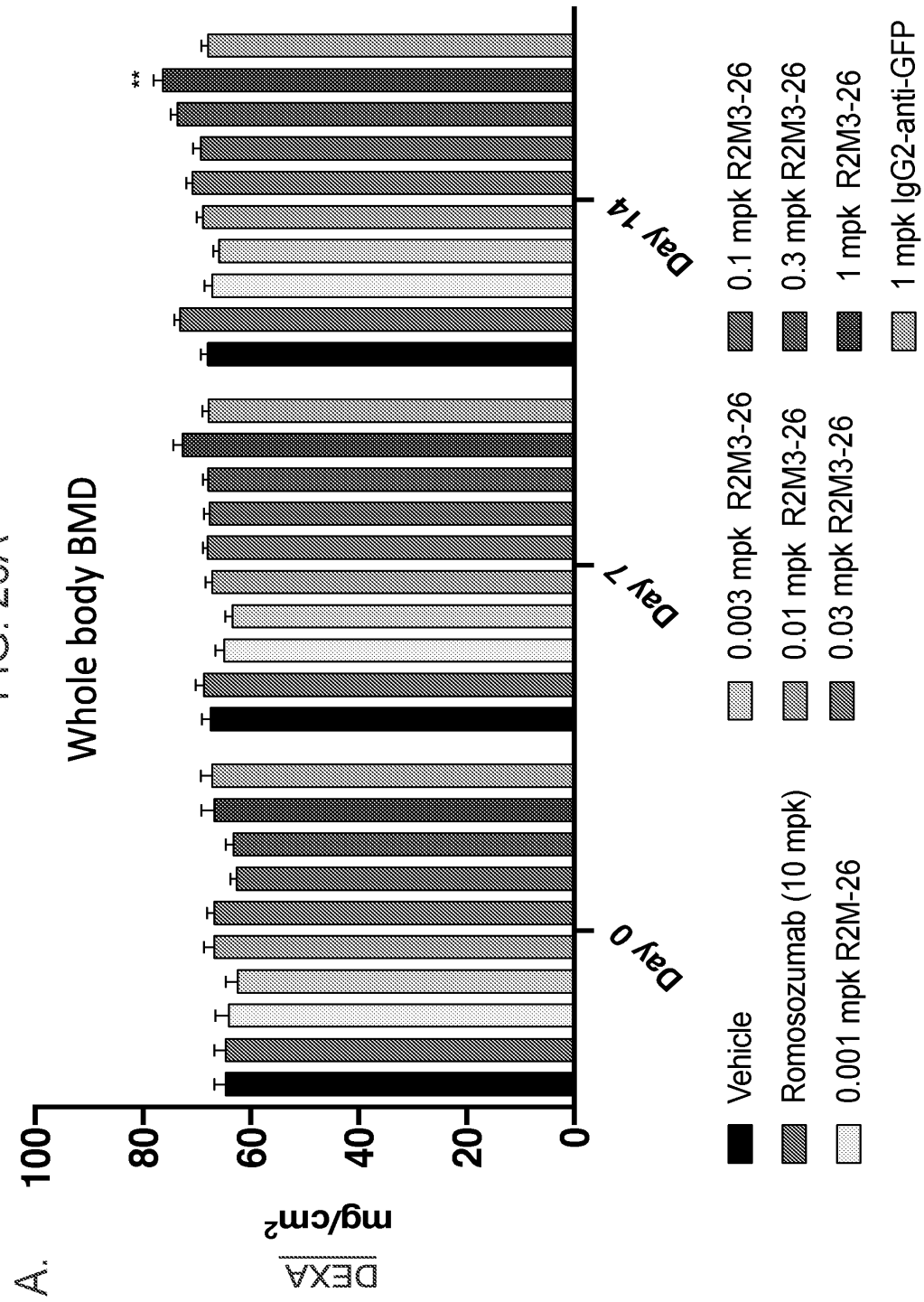

A.
DEXA
Whole body BMD
mg/cm²
100
80
60
40
20
0
Day 0
Day 7
Day 14
**
Vehicle
Romosozumab (10 mpk)
0.001 mpk R2M-26
0.003 mpk R2M3-26
0.01 mpk R2M3-26
0.03 mpk R2M3-26
0.1 mpk R2M3-26
0.3 mpk R2M3-26
1 mpk R2M3-26
1 mpk IgG2-anti-GFP

FIG. 26B

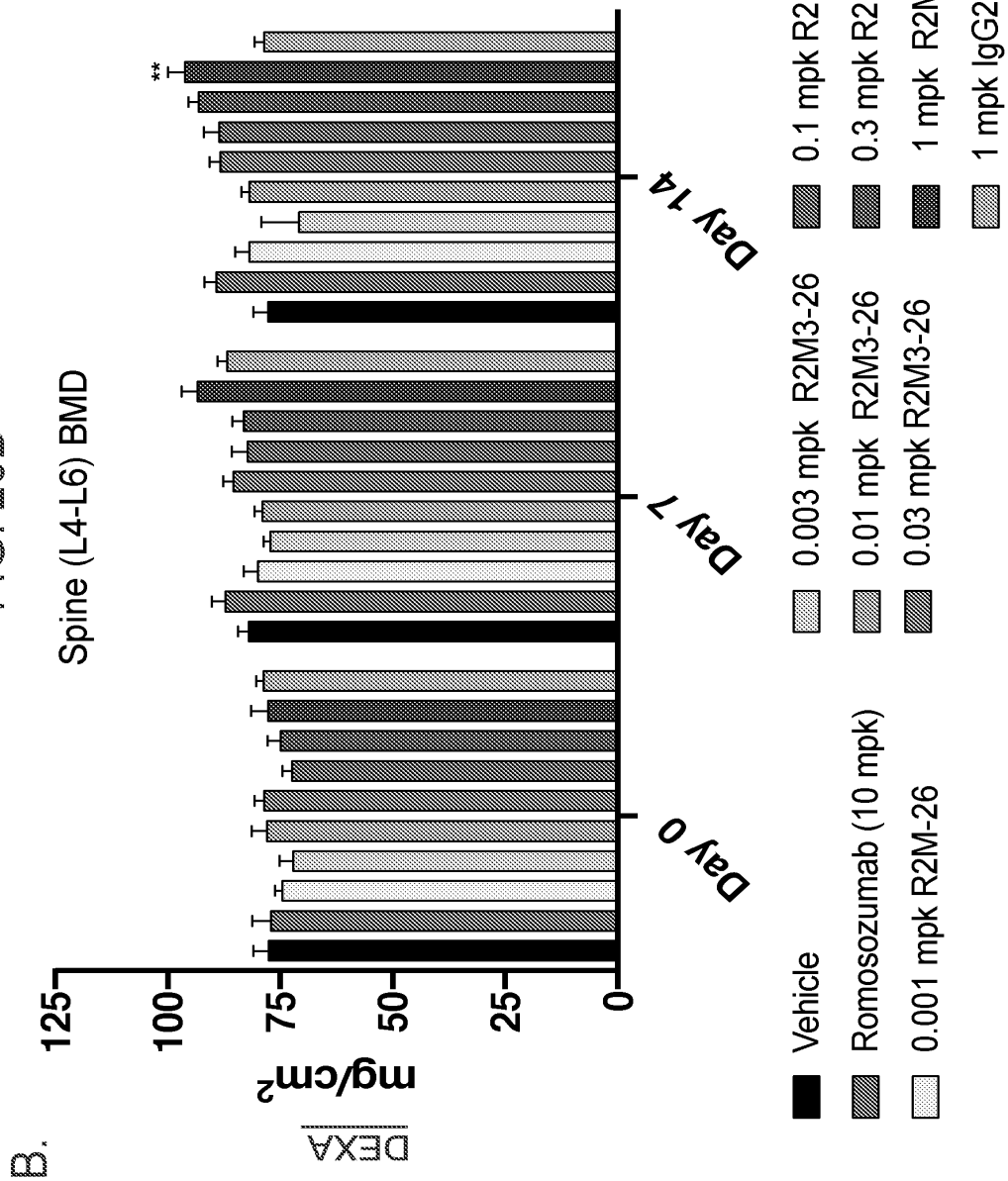

B.
Spine (L4-L6) BMD
DEXA
mg/cm²
125
100
75
50
25
0
Day 0
Day 7
Day 14
**
Vehicle
Romosozumab (10 mpk)
0.001 mpk R2M-26
0.003 mpk R2M3-26
0.01 mpk R2M3-26
0.03 mpk R2M3-26
0.1 mpk R2M3-26
0.3 mpk R2M3-26
1 mpk R2M3-26
1 mpk IgG2-anti-GFP

FIG. 26C

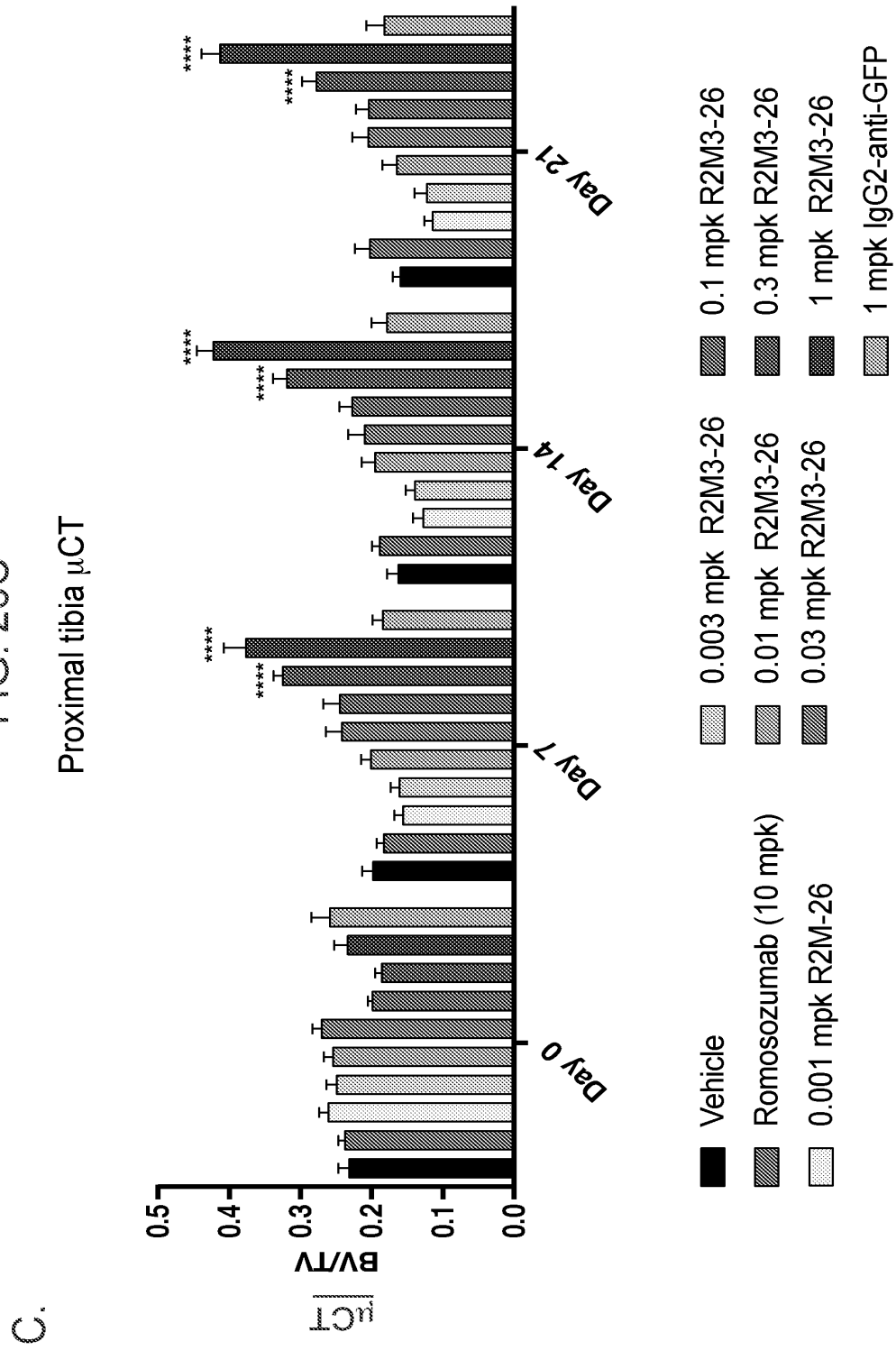

C.
μCT
Proximal tibia μCT
BV/TV
0.0
0.1
0.2
0.3
0.4
0.5
Day 0
Day 7
Day 14
Day 21
****
Vehicle
Romosozumab (10 mpk)
0.001 mpk R2M-26
0.003 mpk R2M3-26
0.01 mpk R2M3-26
0.03 mpk R2M3-26
0.1 mpk R2M3-26
0.3 mpk R2M3-26
1 mpk R2M3-26
1 mpk IgG2-anti-GFP

FIG. 26D

D.

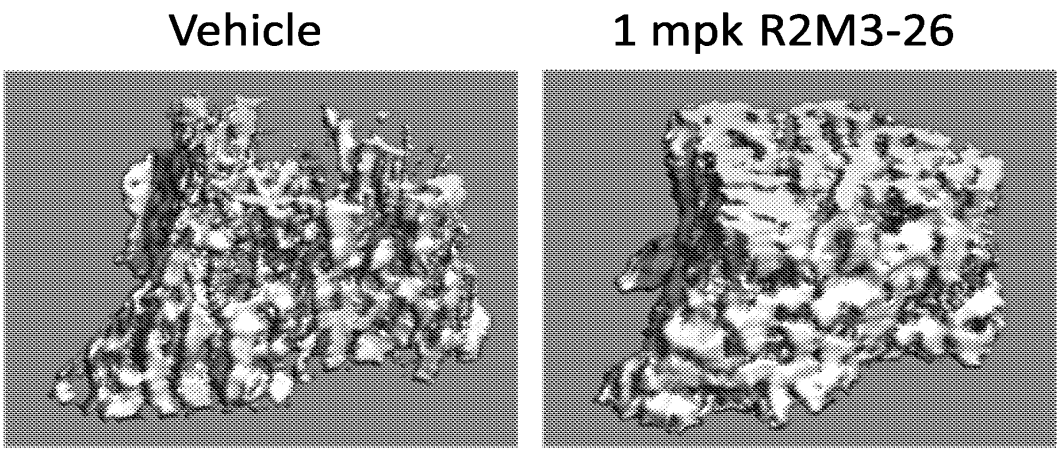
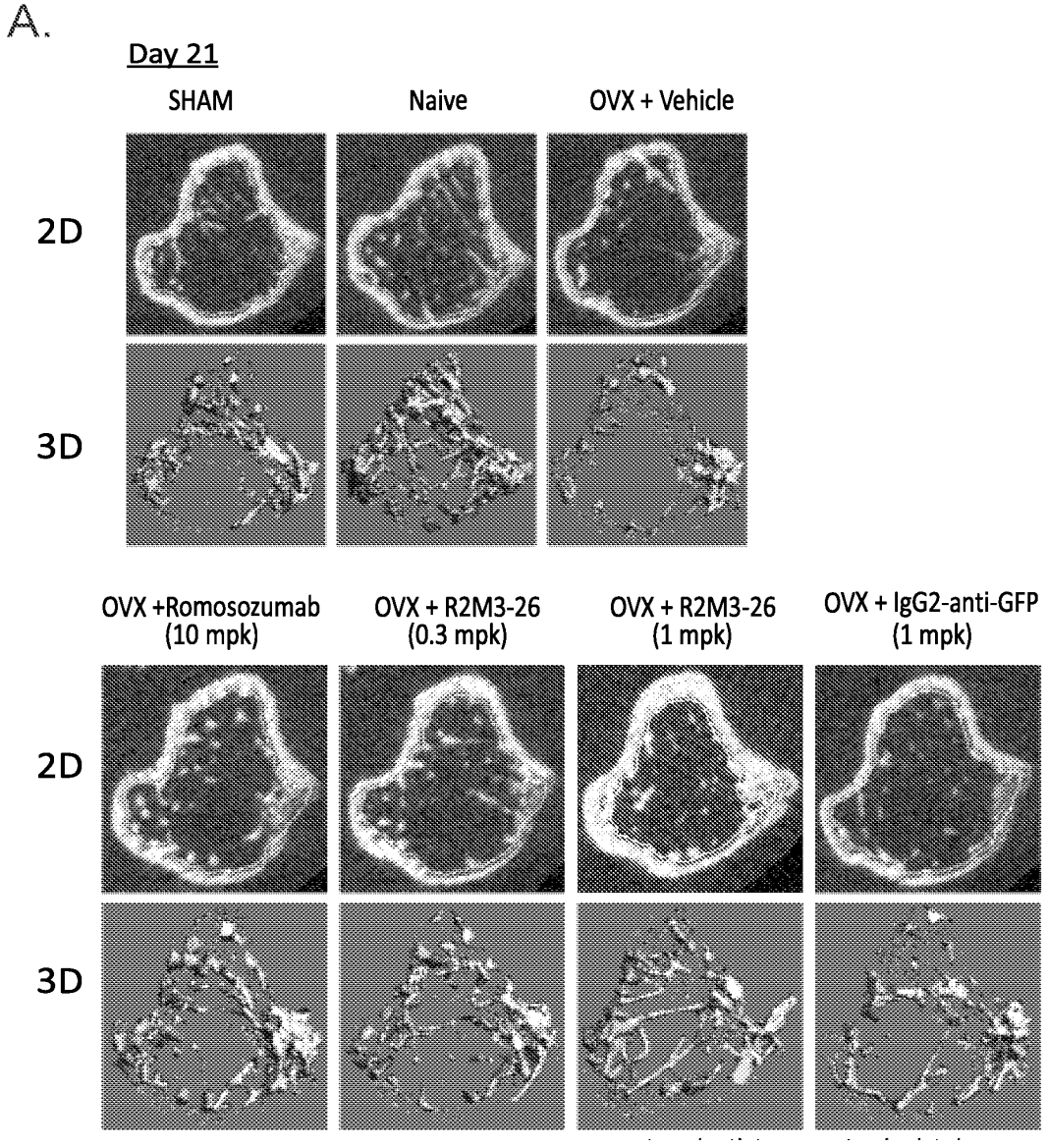


FIG. 27A


A.
Day 21
SHAM
Naive
OVX + Vehicle
2D
3D
OVX +Romosozumab (10 mpk)
OVX + R2M3-26 (0.3 mpk)
OVX + R2M3-26 (1 mpk)
OVX + IgG2-anti-GFP (1 mpk)
2D
3D

B.

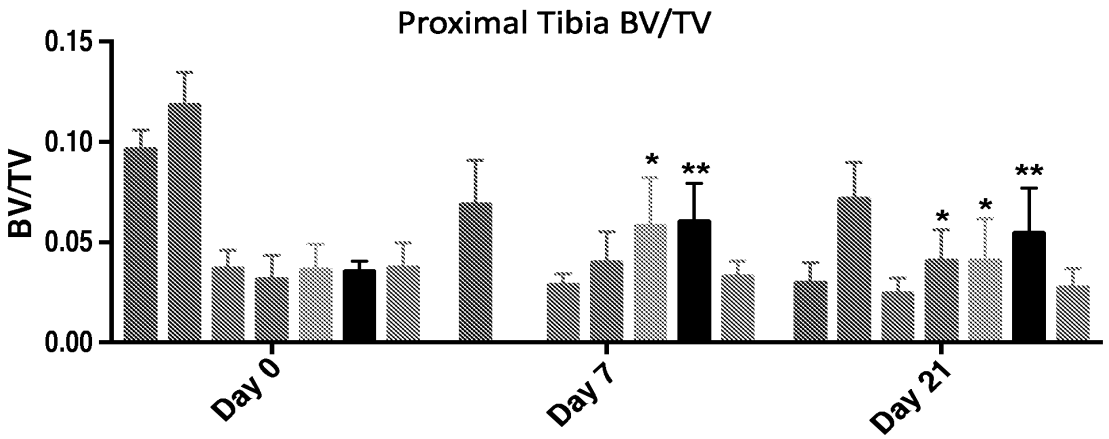


FIG. 27B

C.

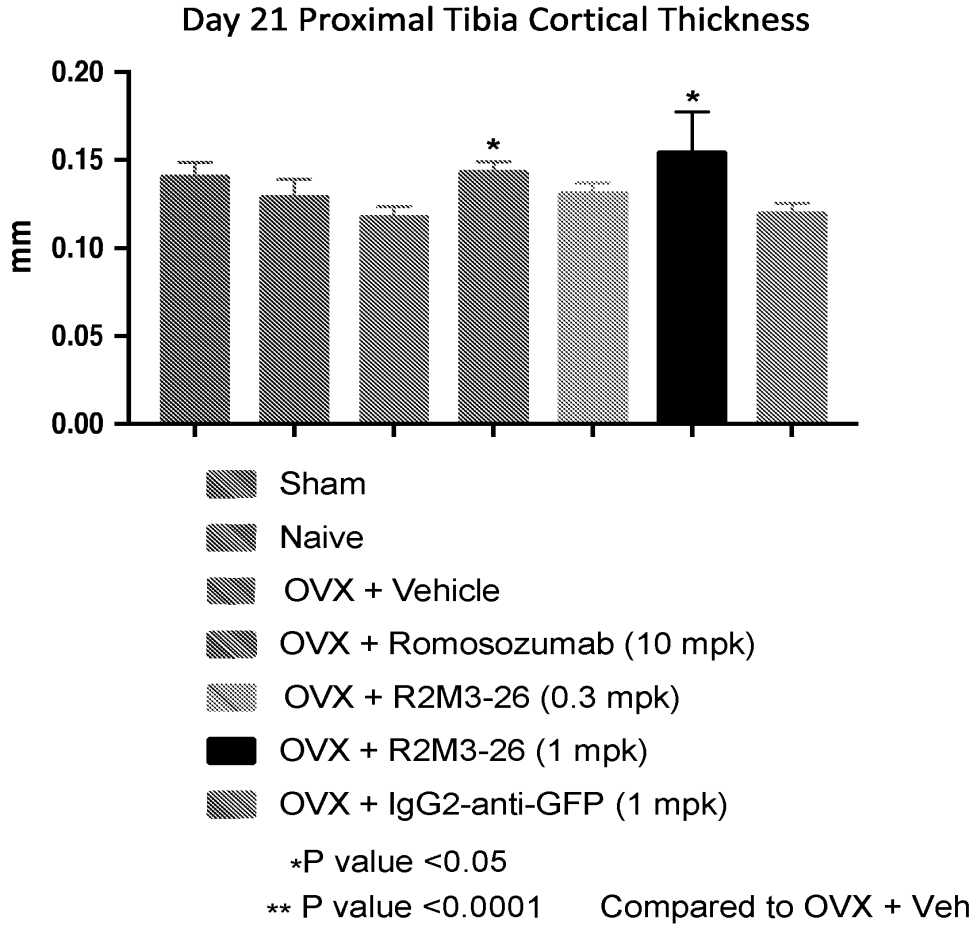
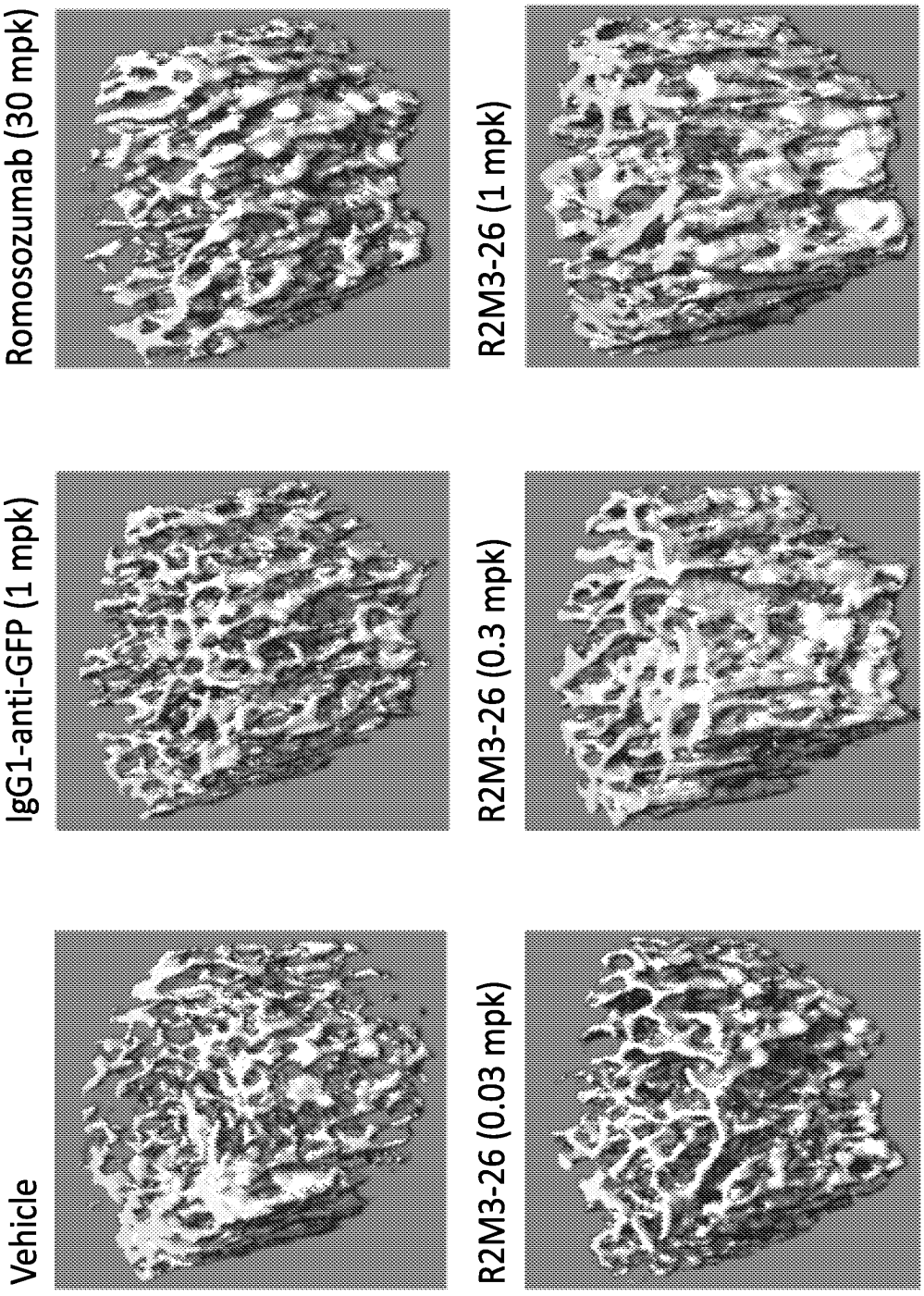


FIG. 27C

FIG. 28A

A.


Vehicle
IgG1-anti-GFP (1 mpk)
Romosozumab (30 mpk)
R2M3-26 (0.03 mpk)
R2M3-26 (0.3 mpk)
R2M3-26 (1 mpk)

B.

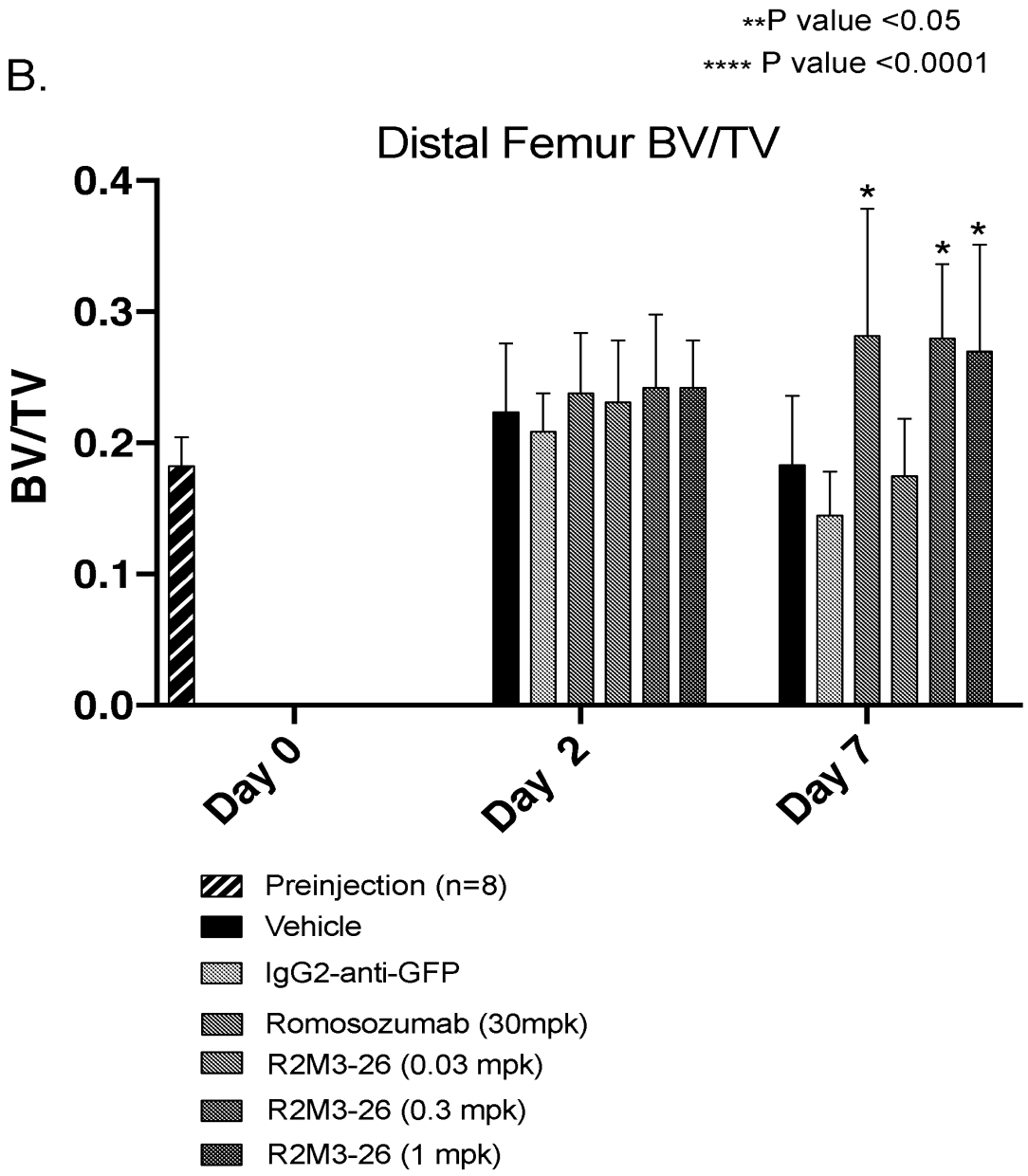

**P value <0.05
**** P value <0.0001
Distal Femur BV/TV
BV/TV
0.4
0.3
0.2
0.1
0.0
*
*
*
Day 0
Day 2
Day 7
Preinjection (n=8)
Vehicle
IgG2-anti-GFP
Romosozumab (30mpk)
R2M3-26 (0.03 mpk)
R2M3-26 (0.3 mpk)
R2M3-26 (1 mpk)

FIG. 28B

C.

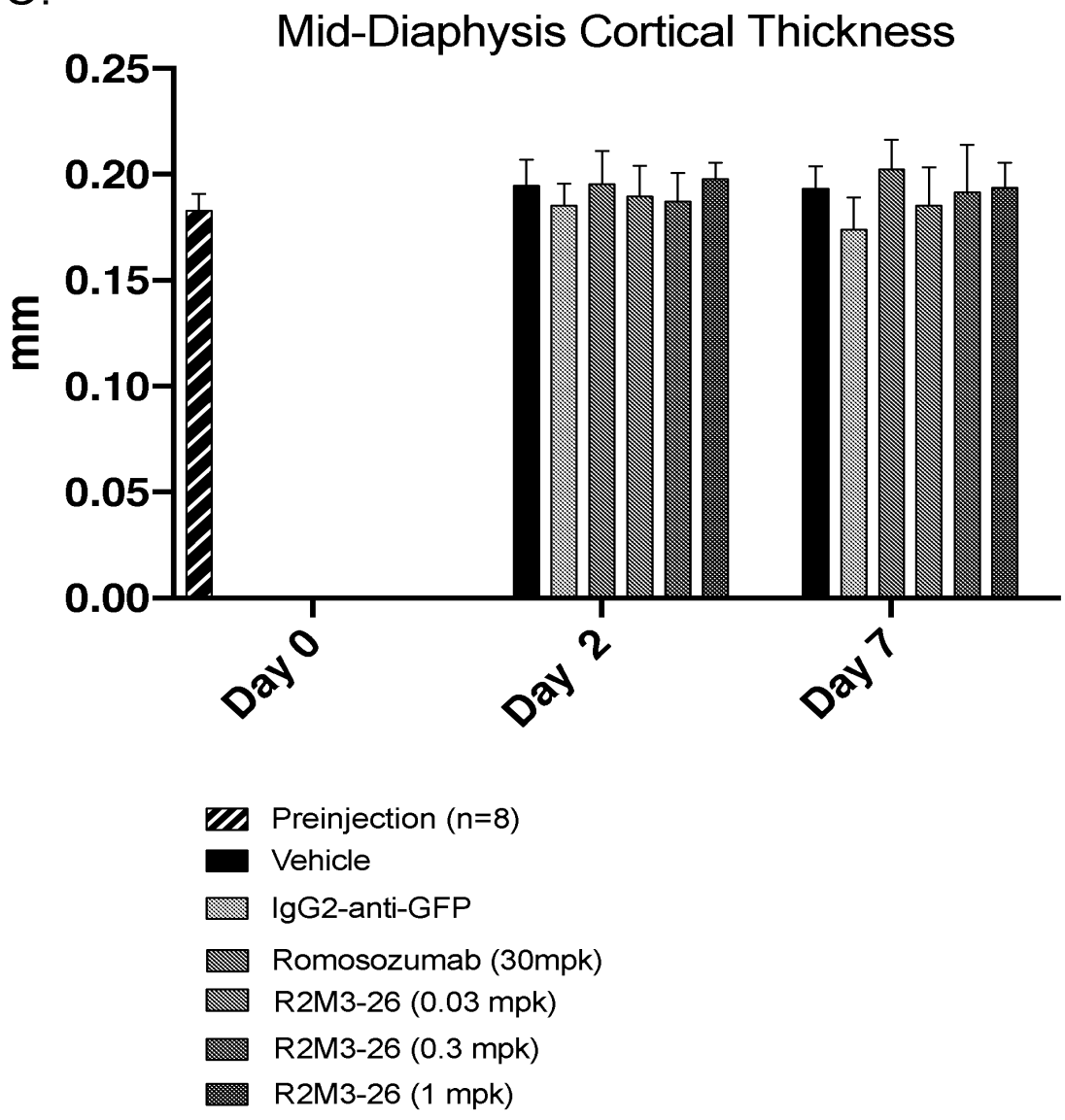

Mid-Diaphysis Cortical Thickness
mm
0.25
0.20
0.15
0.10
0.05
0.00
Day 0
Day 2
Day 7
Preinjection (n=8)
Vehicle
IgG2-anti-GFP
Romosozumab (30mpk)
R2M3-26 (0.03 mpk)
R2M3-26 (0.3 mpk)
R2M3-26 (1 mpk)

FIG. 28C

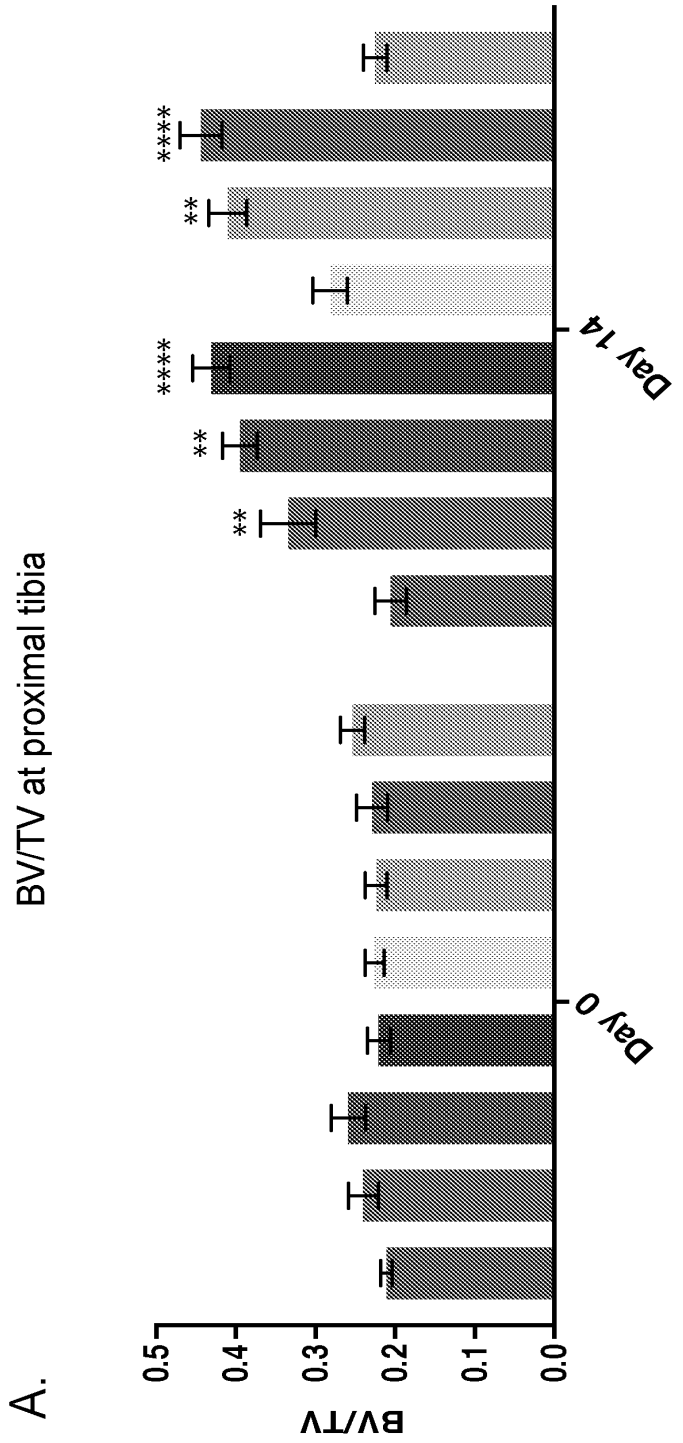


FIG. 29A

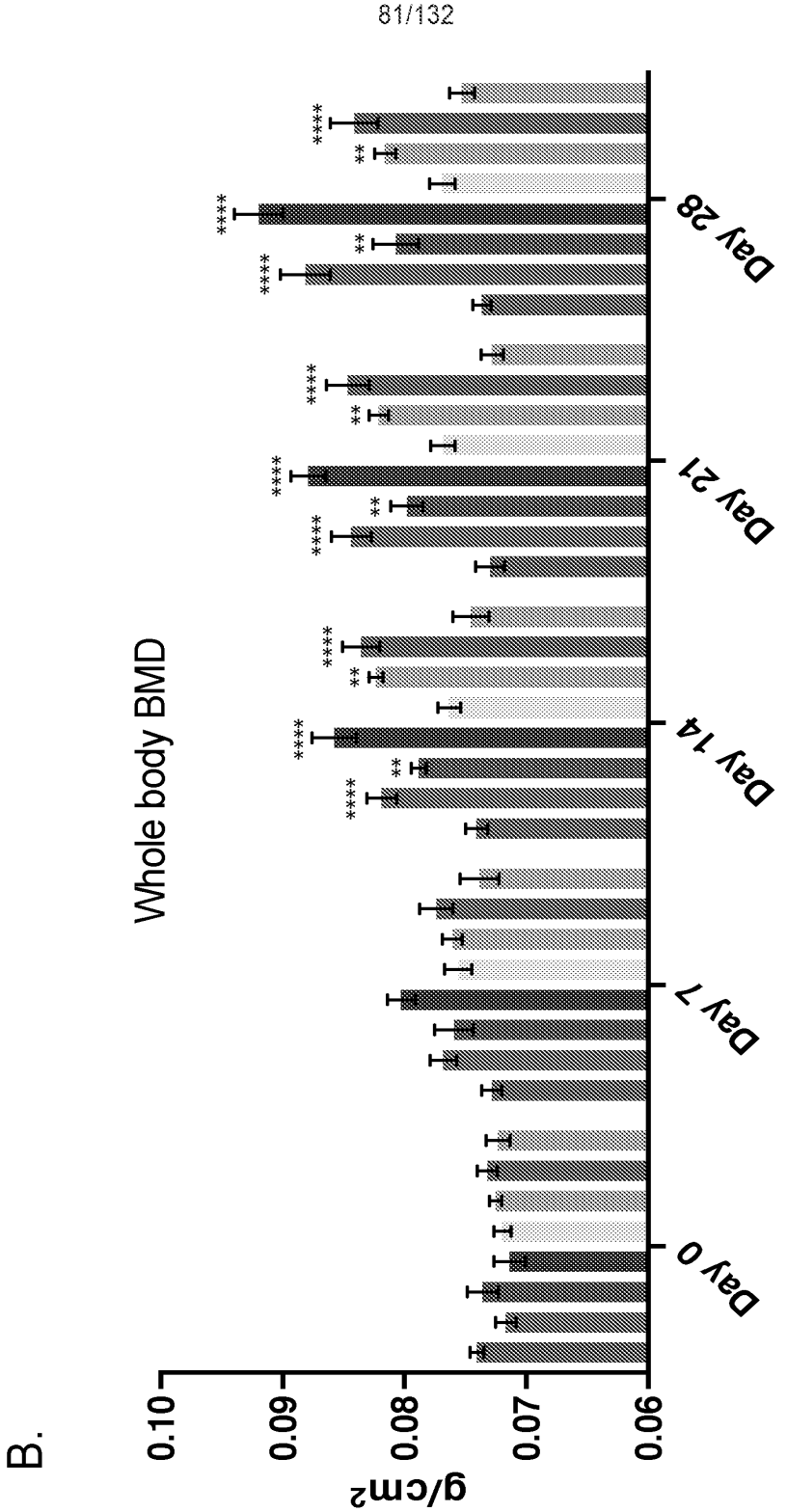

B.
Whole body BMD
g/cm2
0.10
0.09
0.08
0.07
0.06
Day 0
Day 7
Day 14
Day 21
Day 28

FIG. 29B

C.

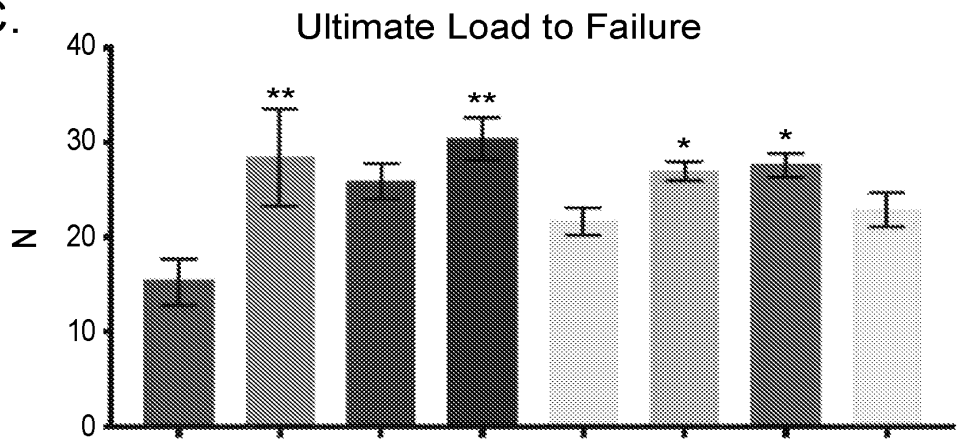


FIG. 29C

D.

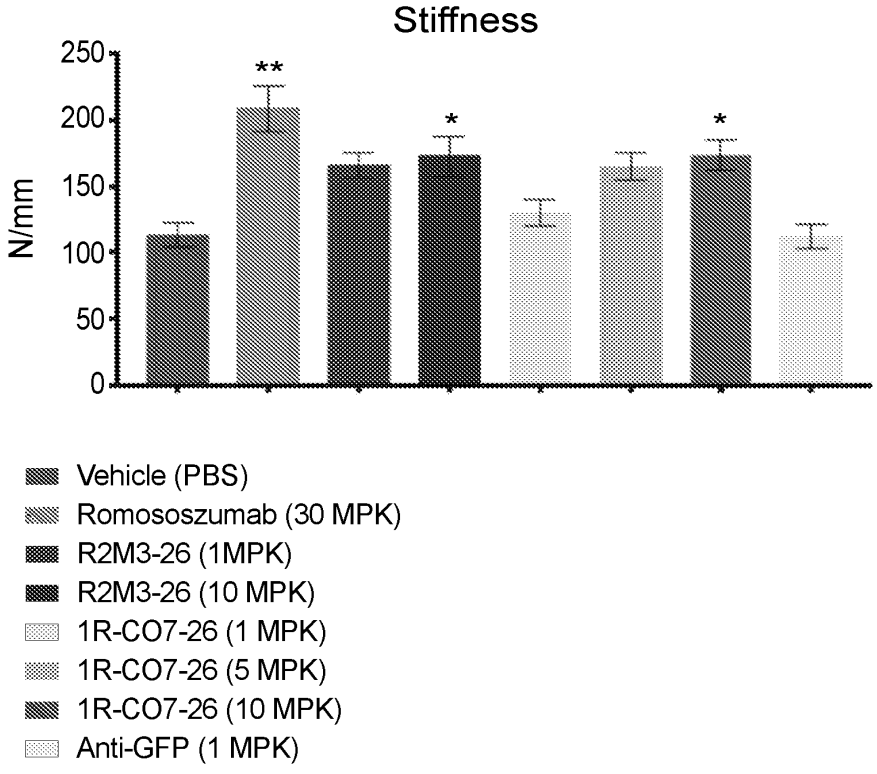


FIG. 29D

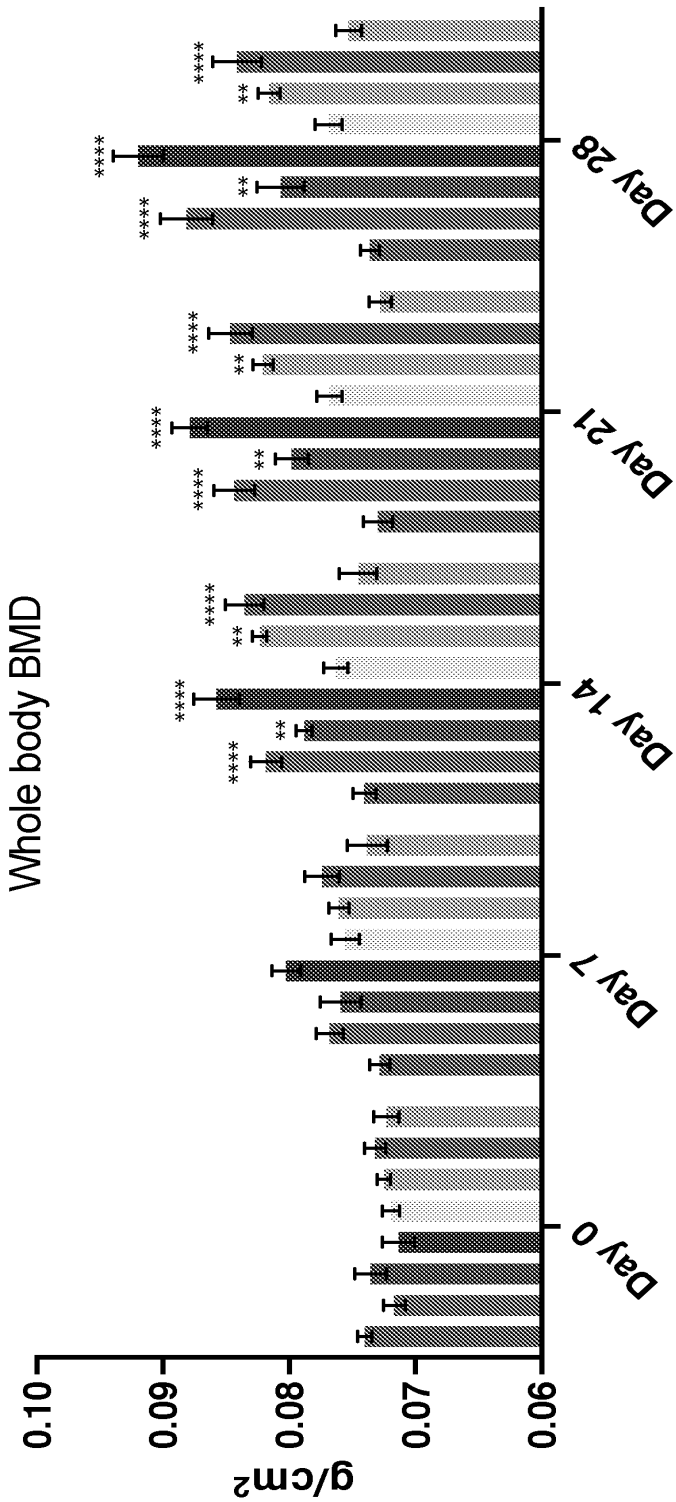
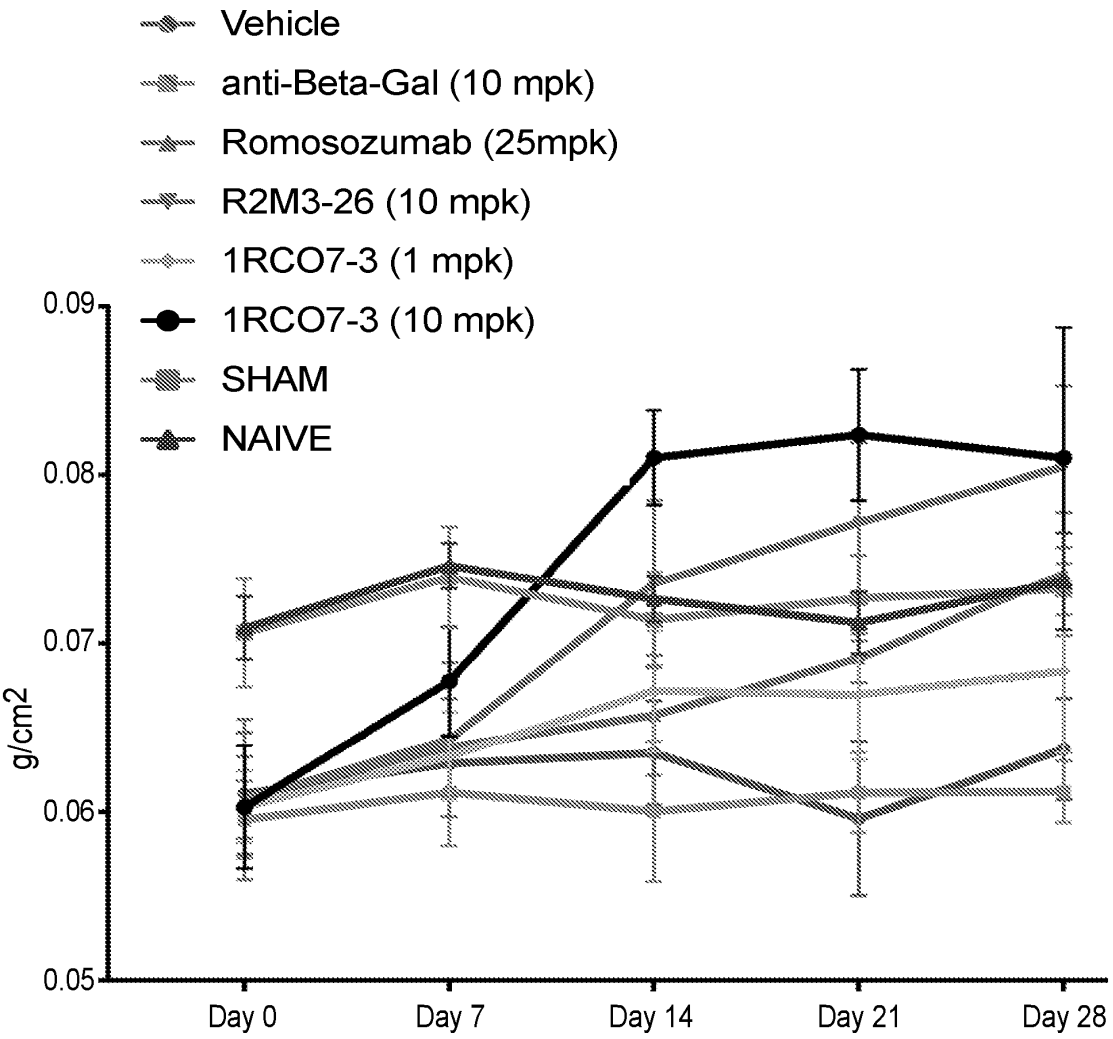

Whole body BMD
g/cm2
0.10
0.09
0.08
0.07
0.06
Day 0
Day 7
Day 14
Day 21
Day 28
Vehicle (PBS)
Romosozumab (30 MPK)
R2M3-26 (1MPK)
R2M3-26 (10 MPK)
1R-CO7-26 (1 MPK)
1R-CO7-26 (5 MPK)
1R-CO7-26 (10 MPK)
Anti-GFP (1 MPK)

FIG. 30

FIG. 31


Vehicle
anti-Beta-Gal (10 mpk)
Romosozumab (25mpk)
R2M3-26 (10 mpk)
1RCO7-3 (1 mpk)
1RCO7-3 (10 mpk)
SHAM
NAIVE
0.09
0.08
0.07
0.06
0.05
g/cm2
Day 0
Day 7
Day 14
Day 21
Day 28

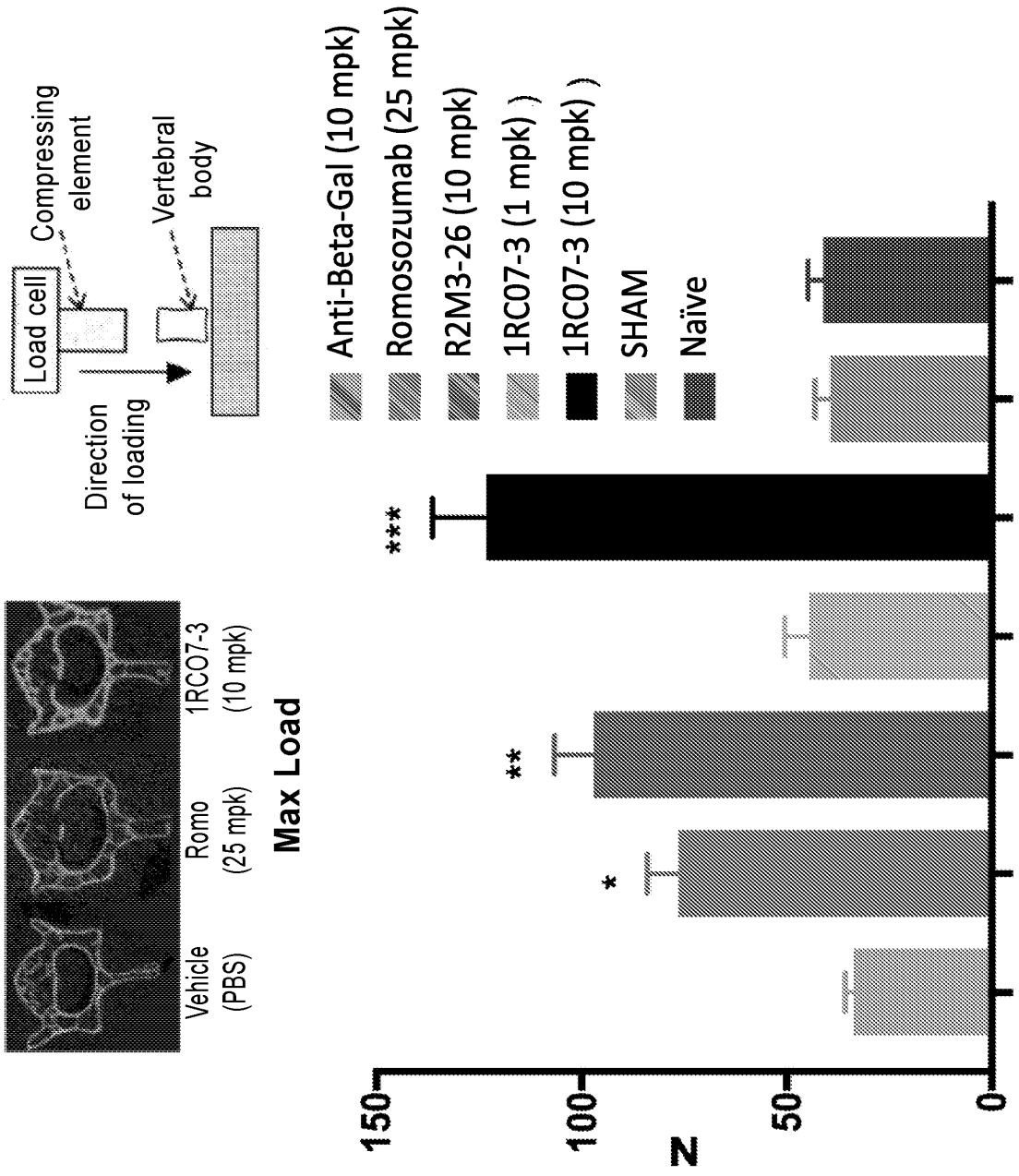

Load cell
Compressing element
Direction of loading
Vertebral body
Vehicle (PBS)
Romo (25 mpk)
1RC07-3 (10 mpk)
Max Load
Anti-Beta-Gal (10 mpk)
Romosozumab (25 mpk)
R2M3-26 (10 mpk)
1RC07-3 (1 mpk))
1RC07-3 (10 mpk))
SHAM
Naïve
***
**
*
N
150
100
50
0

FIG. 32

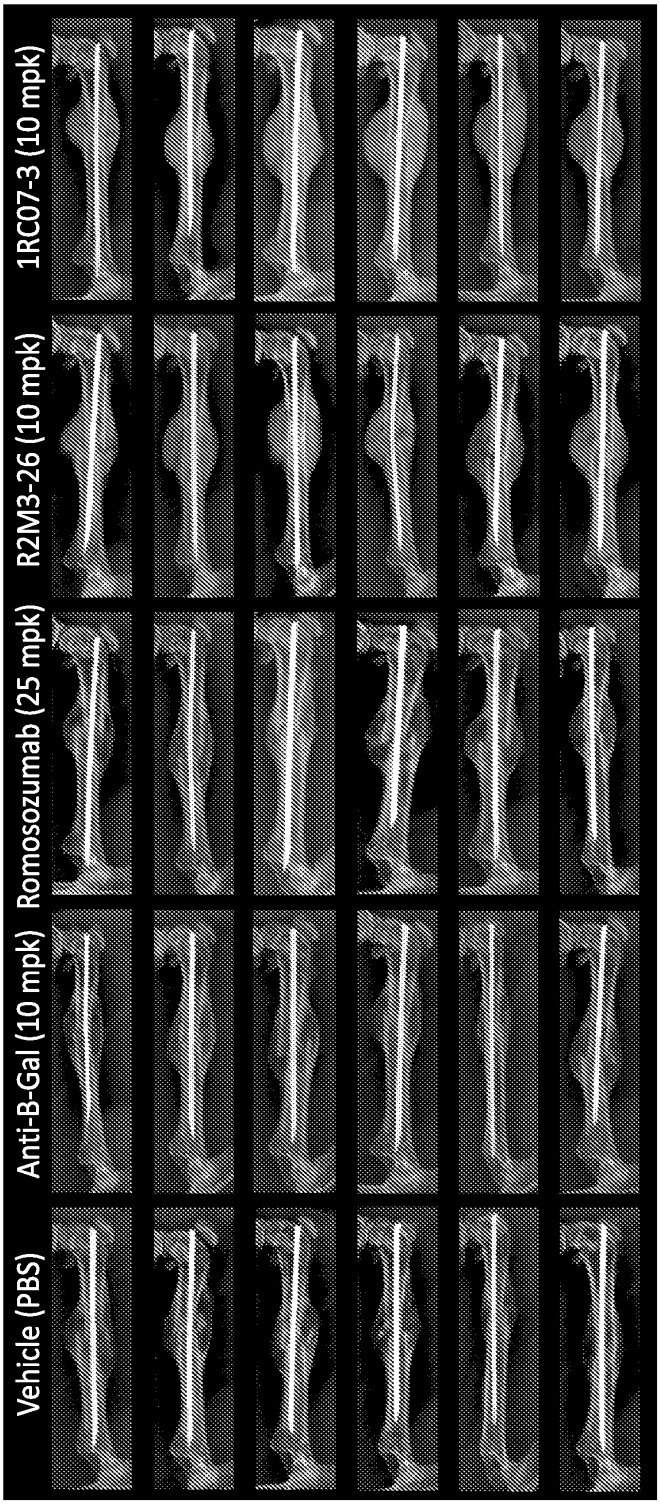


FIG. 33A *1 week of treatment*

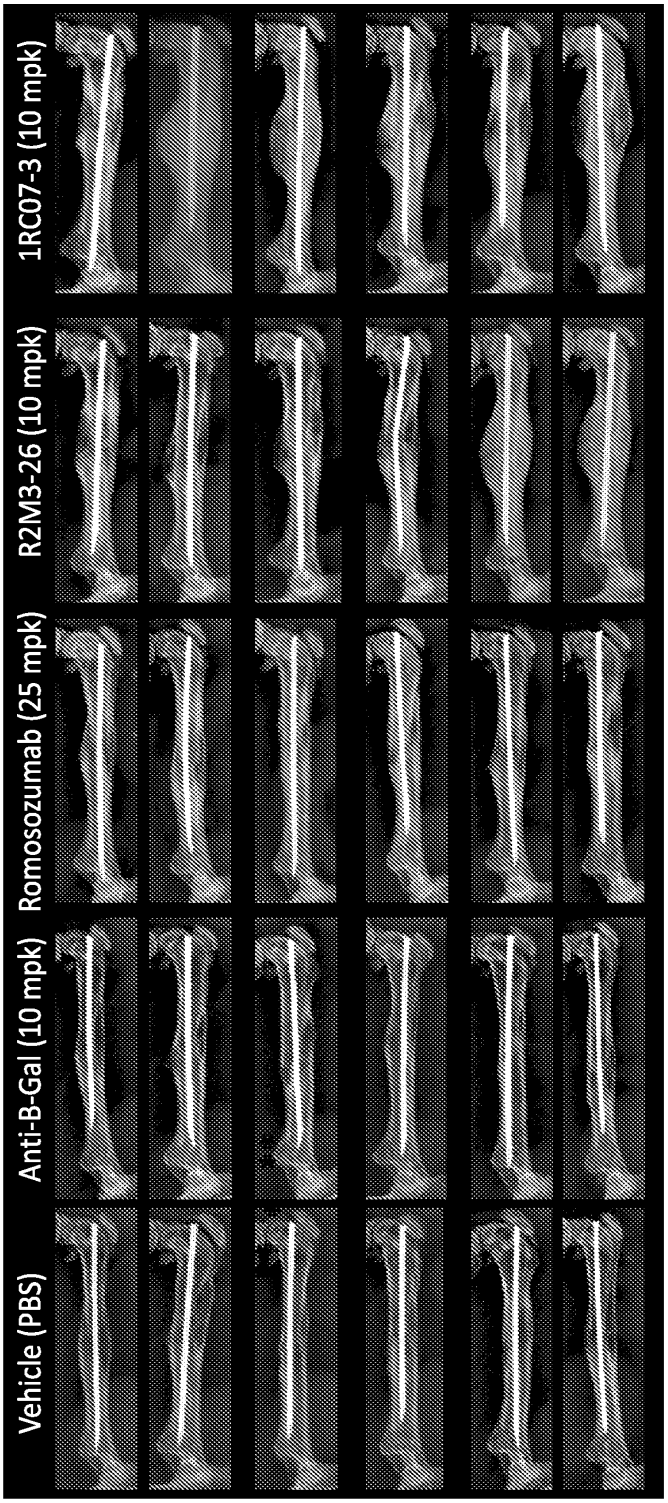
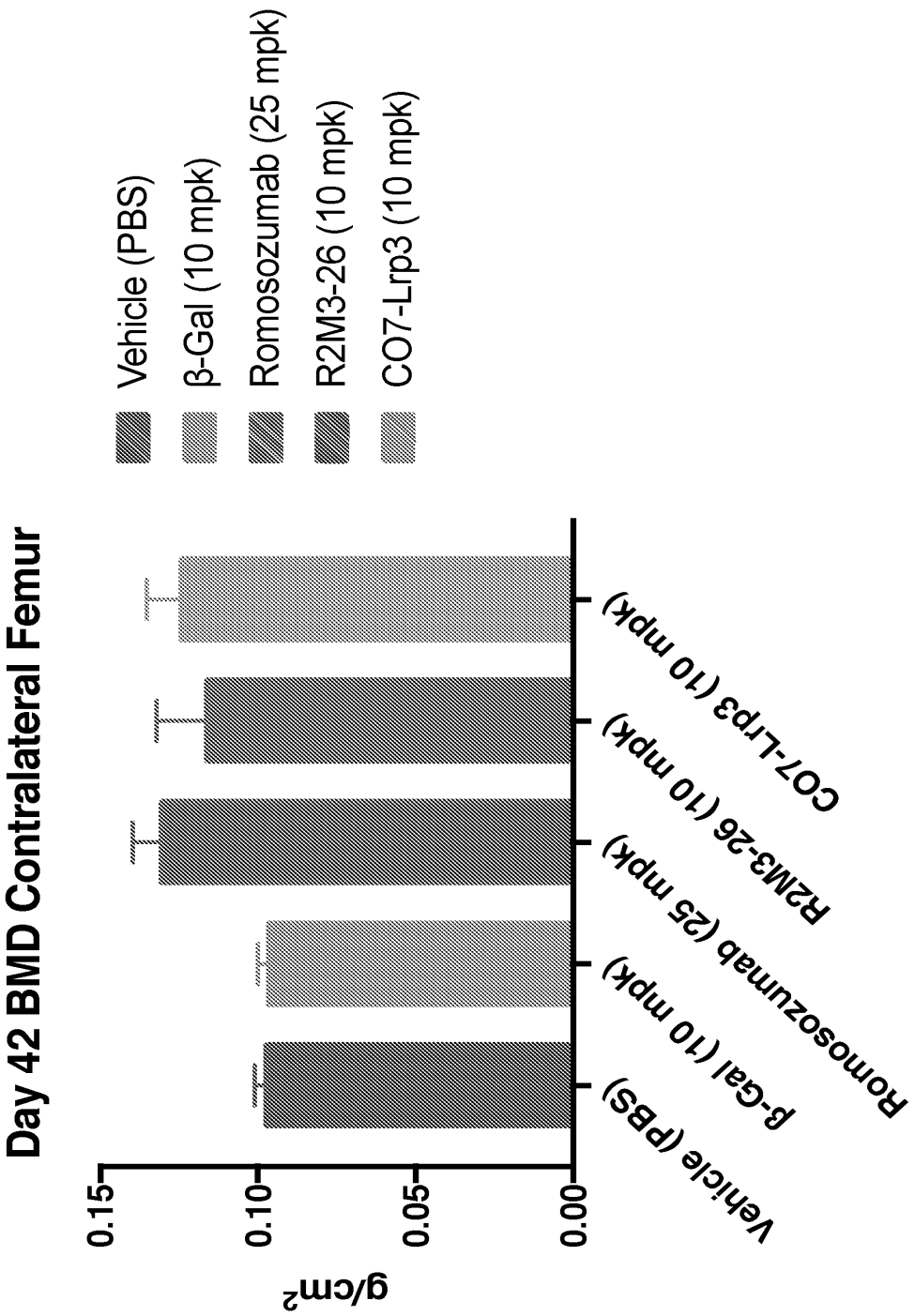


FIG. 33B *6 week of treatment*

FIG. 33C


Day 42 BMD Contralateral Femur
g/cm²
0.15
0.10
0.05
0.00
Vehicle (PBS)
β-Gal (10 mpk)
Romosozumab (25 mpk)
R2M3-26 (10 mpk)
CO7-Lrp3 (10 mpk)
Vehicle (PBS)
β-Gal (10 mpk)
Romosozumab (25 mpk)
R2M3-26 (10 mpk)
CO7-Lrp3 (10 mpk)

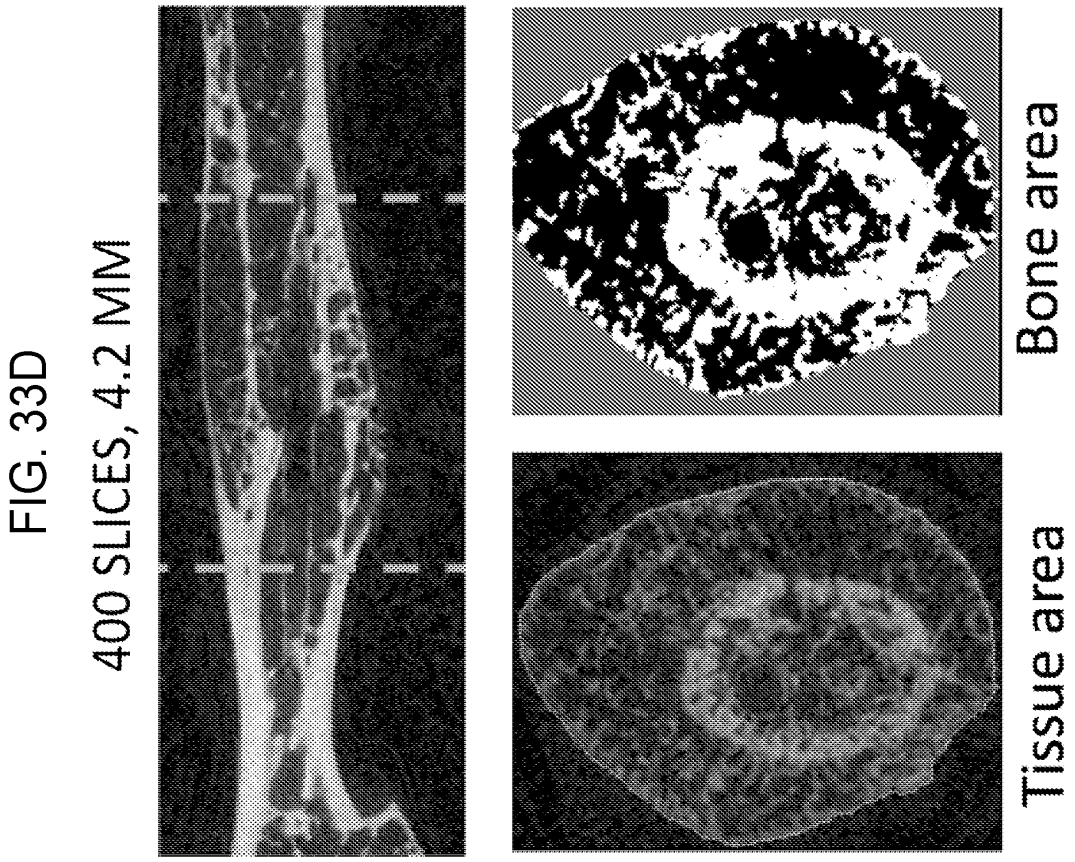


FIG. 33D

FIG. 33D (contd.)

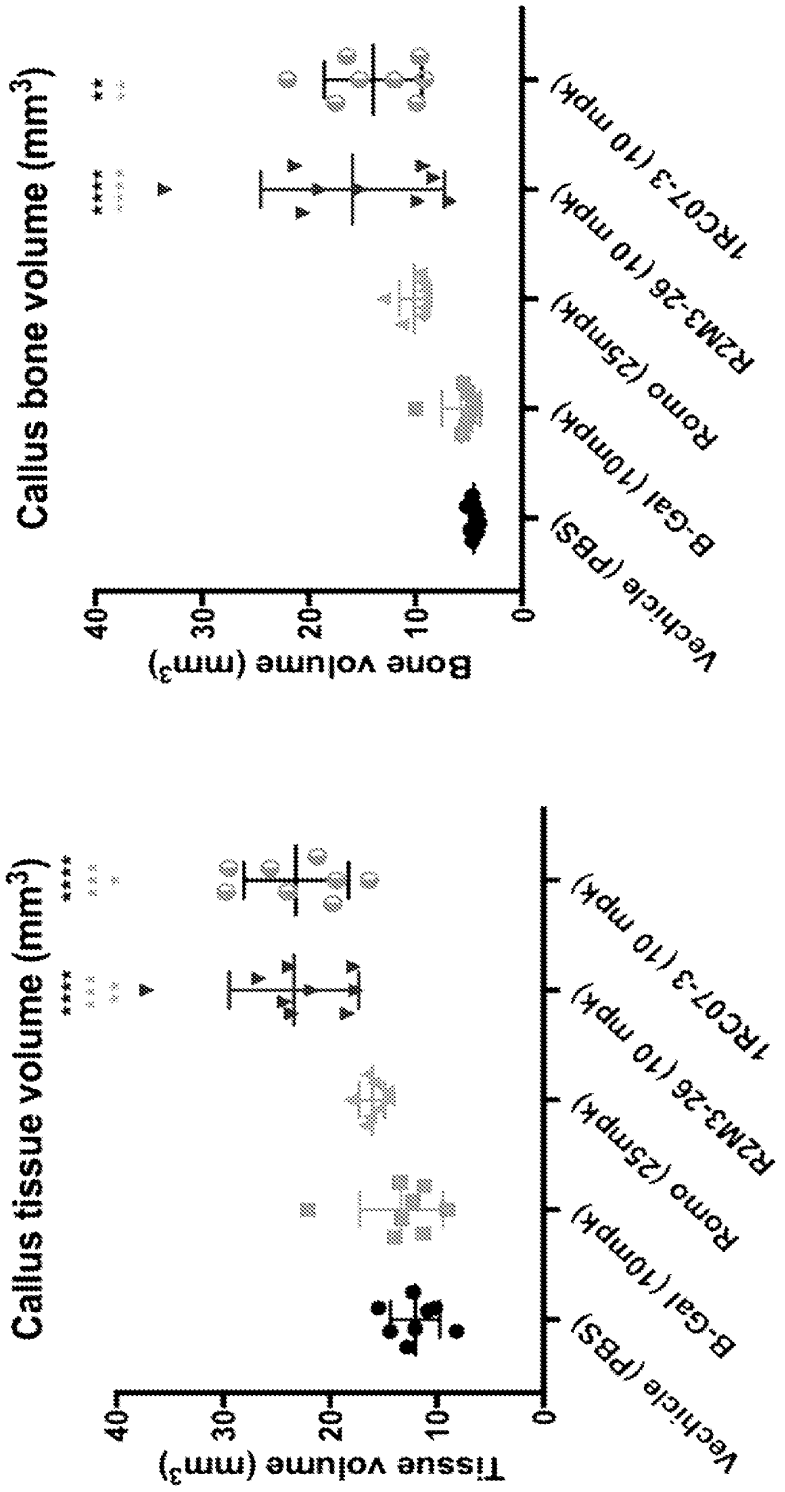


FIG. 33D (contd.)

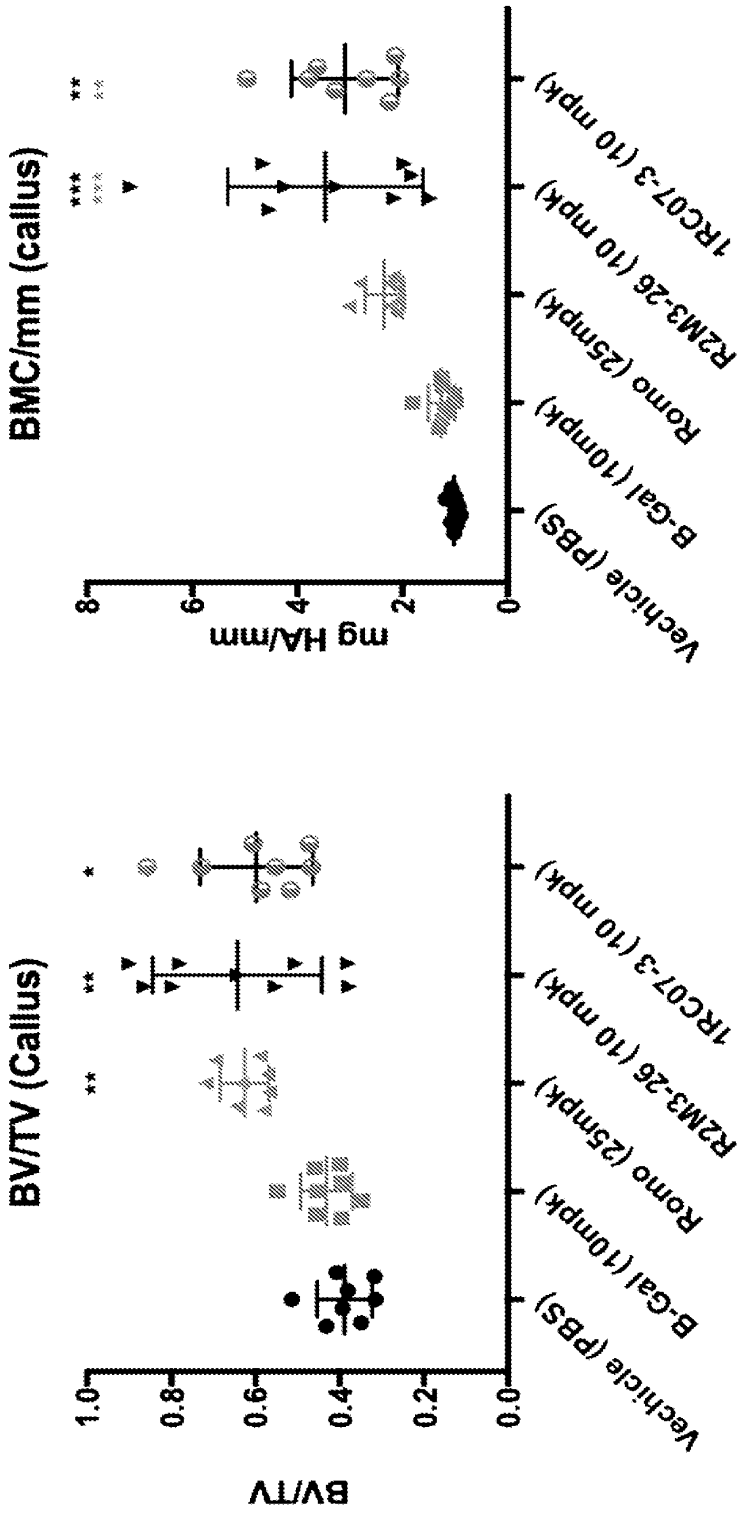


FIG. 33D (contd.)

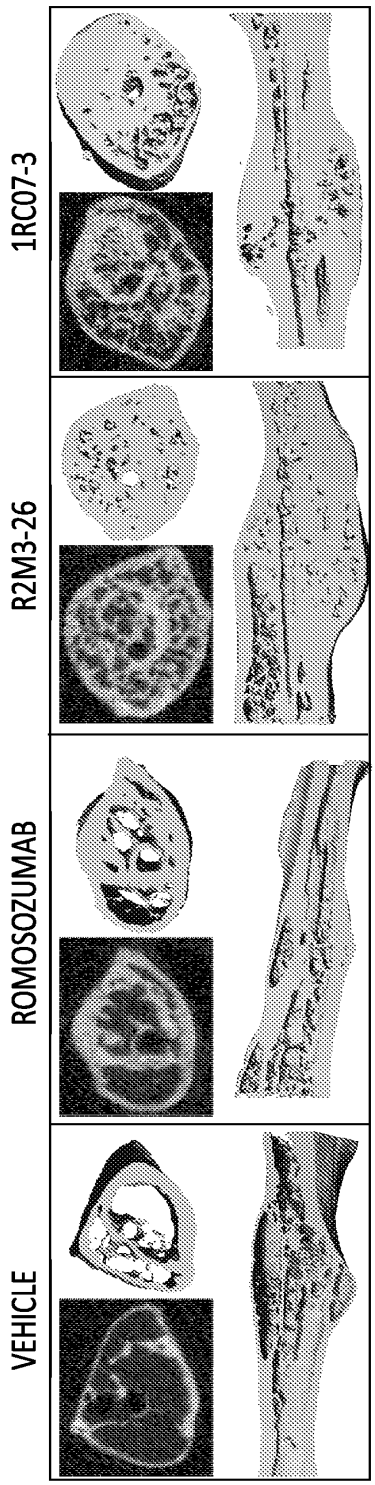


FIG. 34

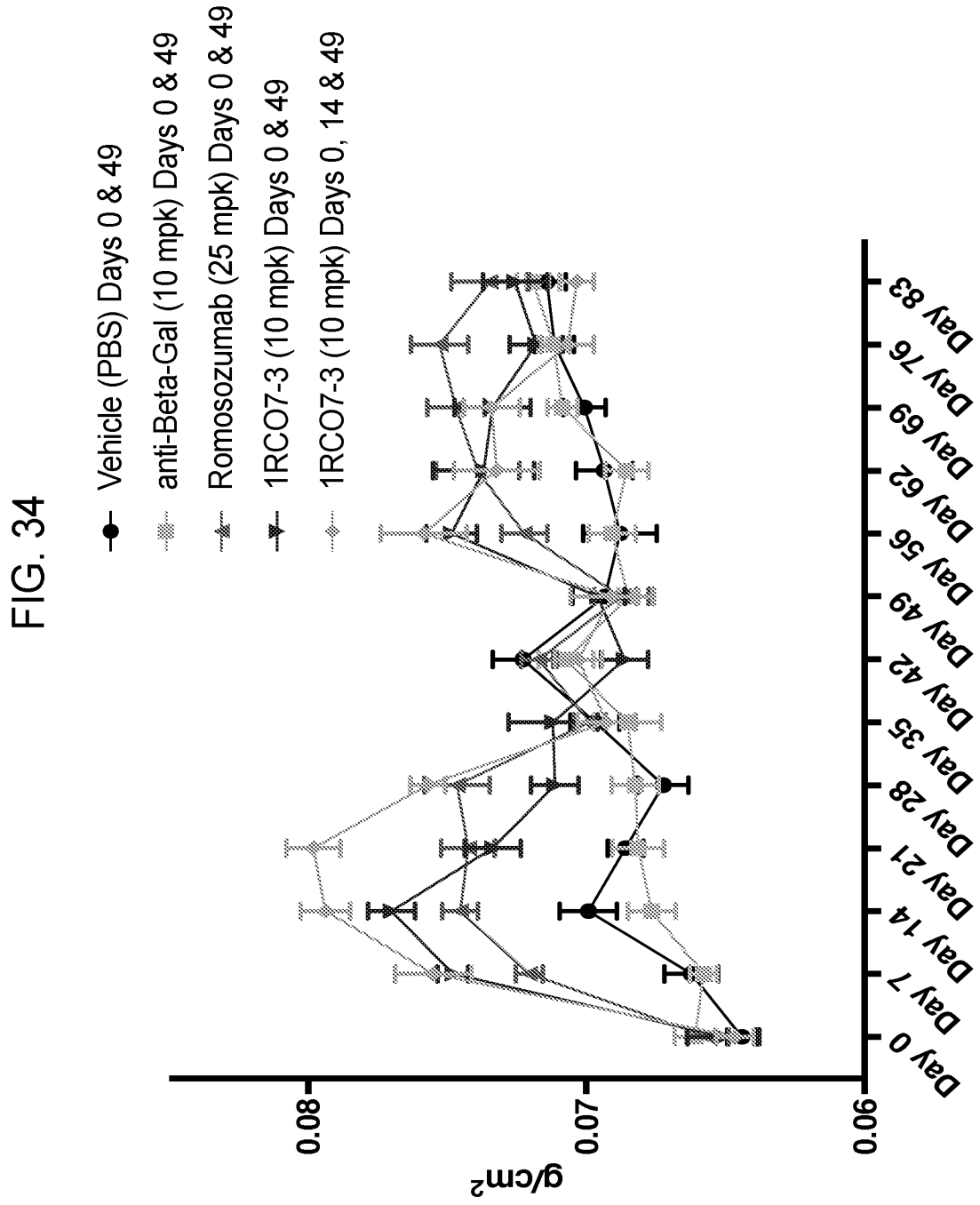

Vehicle (PBS) Days 0 & 49
anti-Beta-Gal (10 mpk) Days 0 & 49
Romosozumab (25 mpk) Days 0 & 49
1RCO7-3 (10 mpk) Days 0 & 49
1RCO7-3 (10 mpk) Days 0, 14 & 49
g/cm²
0.08
0.07
0.06
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Day 49
Day 56
Day 62
Day 69
Day 76
Day 83

FIG. 35

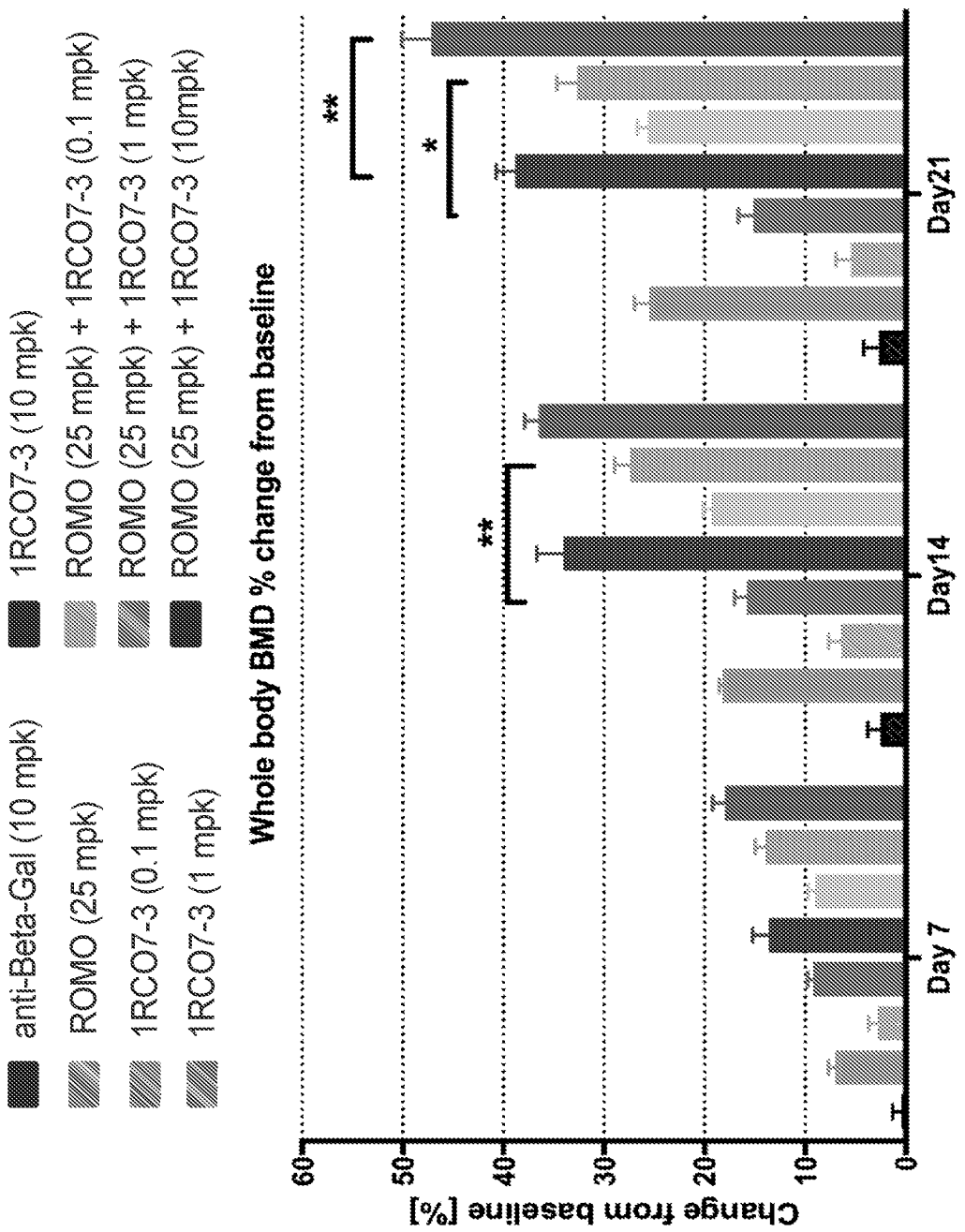
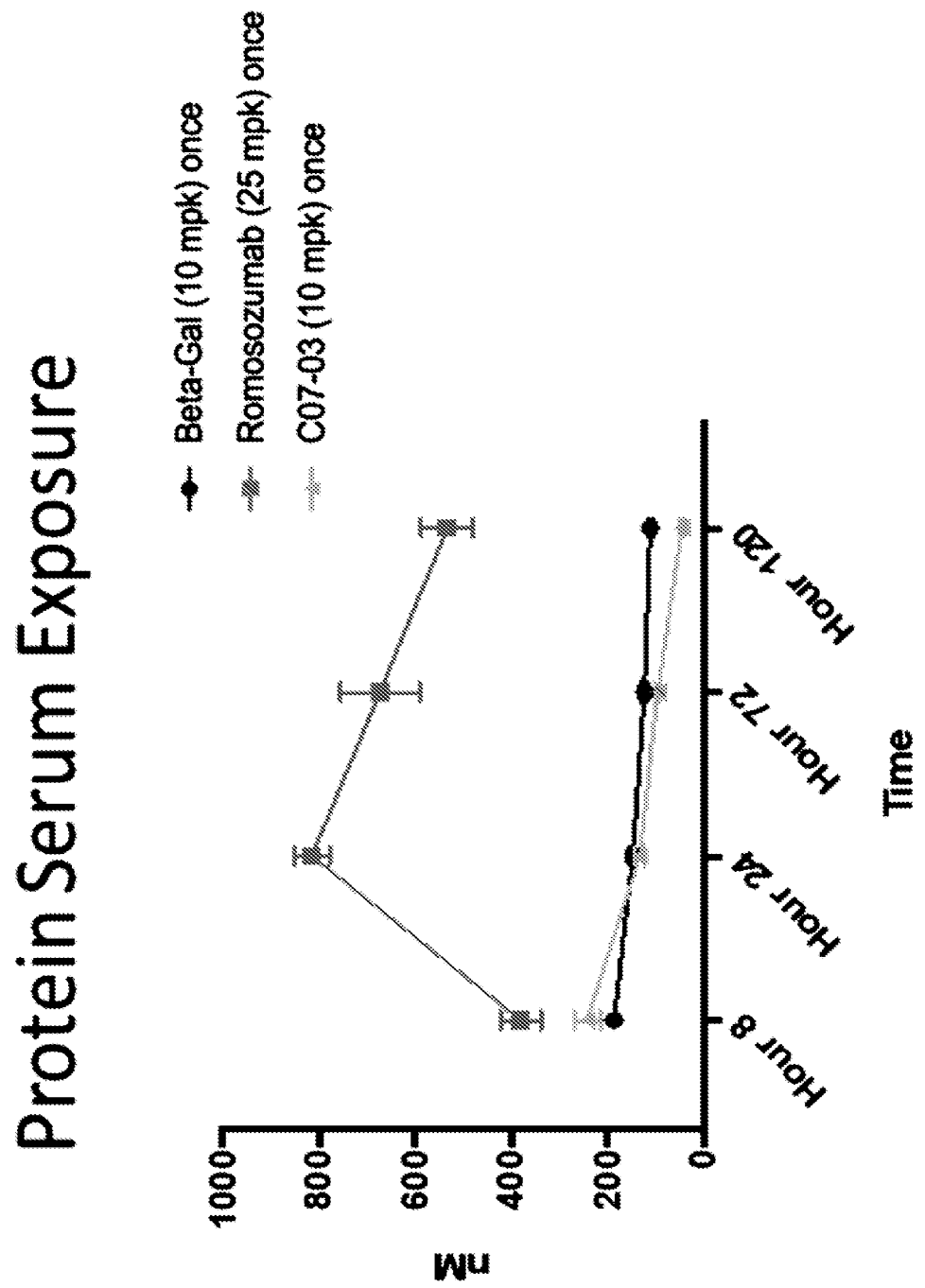

anti-Beta-Gal (10 mpk)
ROMO (25 mpk)
1RCO7-3 (0.1 mpk)
1RCO7-3 (1 mpk)
1RCO7-3 (10 mpk)
ROMO (25 mpk) + 1RCO7-3 (0.1 mpk)
ROMO (25 mpk) + 1RCO7-3 (1 mpk)
ROMO (25 mpk) + 1RCO7-3 (10mpk)
Whole body BMD % change from baseline
Change from baseline [%]
0
10
20
30
40
50
60
Day 7
Day14
Day21
**
*
**

FIG. 36


Protein Serum Exposure
Beta-Gal (10 mpk) once
Romosozumab (25 mpk) once
C07-03 (10 mpk) once
nM
1000
800
600
400
200
0
HOUR 8
HOUR 24
HOUR 72
HOUR 120
Time

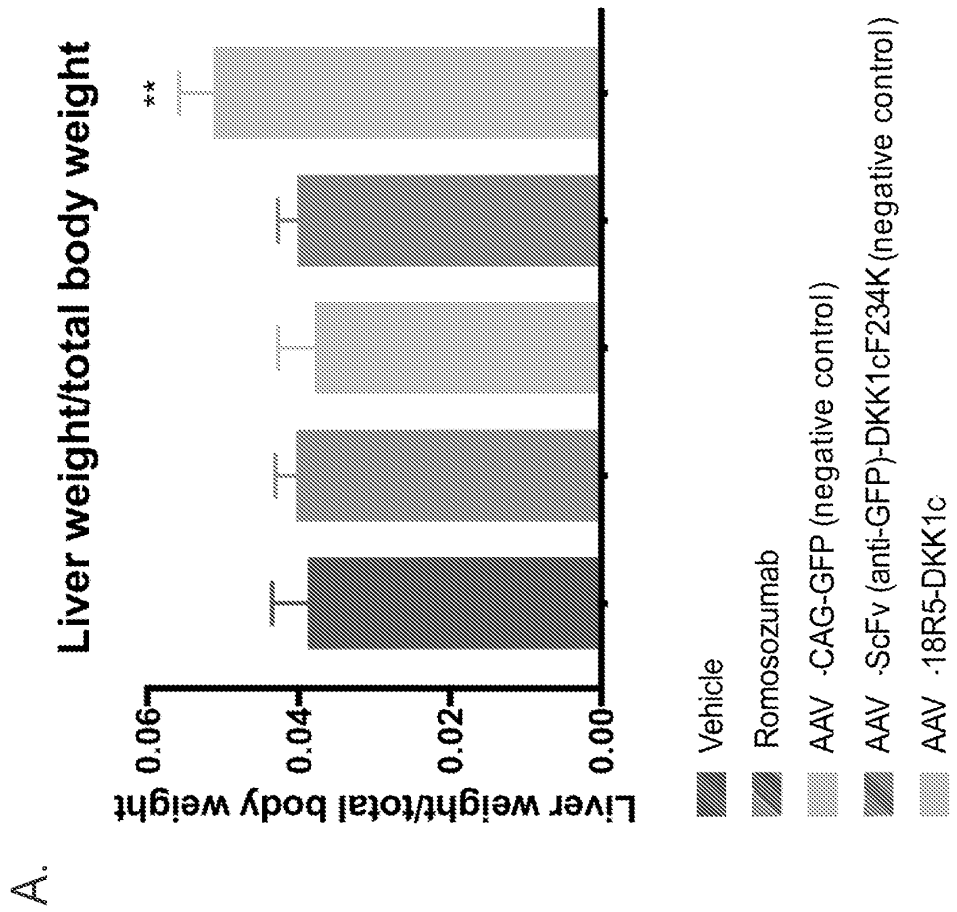

A.
Liver weight/total body weight
Liver weight/total body weight
0.06
0.04
0.02
0.00
**
Vehicle
Romosozumab
AAV -CAG-GFP (negative control)
AAV -ScFv (anti-GFP)-DKK1cF234K (negative control)
AAV -18R5-DKK1c

FIG. 37

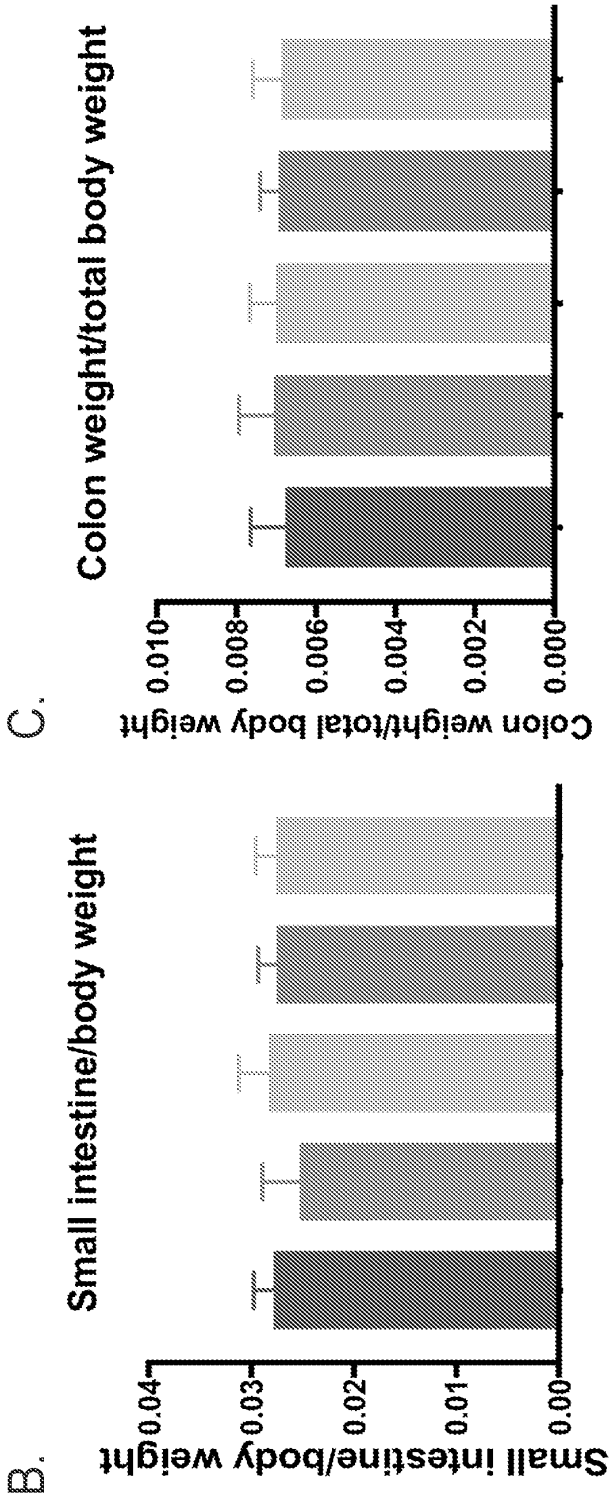
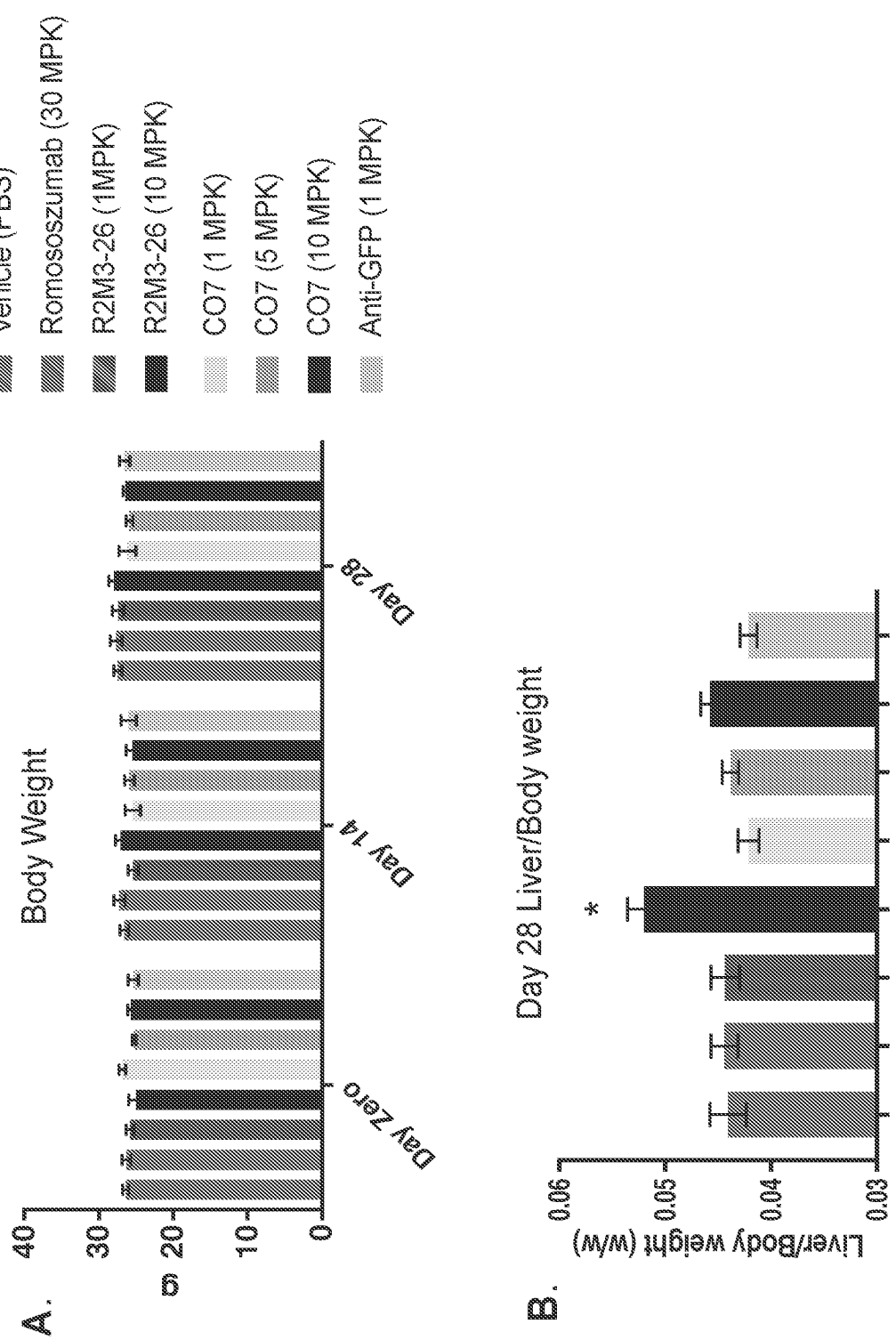

B.
Small intestine/body weight
Small intestine/body weight
0.04
0.03
0.02
0.01
0.00
C.
Colon weight/total body weight
Colon weight/total body weight
0.010
0.008
0.006
0.004
0.002
0.000

FIG. 37 (contd.)

FIG. 38


A.
Body Weight
g
40
30
20
10
0
Day Zero
Day 14
Day 28
Vehicle (PBS)
Romososzumab (30 MPK)
R2M3-26 (1MPK)
R2M3-26 (10 MPK)
CO7 (1 MPK)
CO7 (5 MPK)
CO7 (10 MPK)
Anti-GFP (1 MPK)
B.
Day 28 Liver/Body weight
*
Liver/Body weight (w/w)
0.06
0.05
0.04
0.03

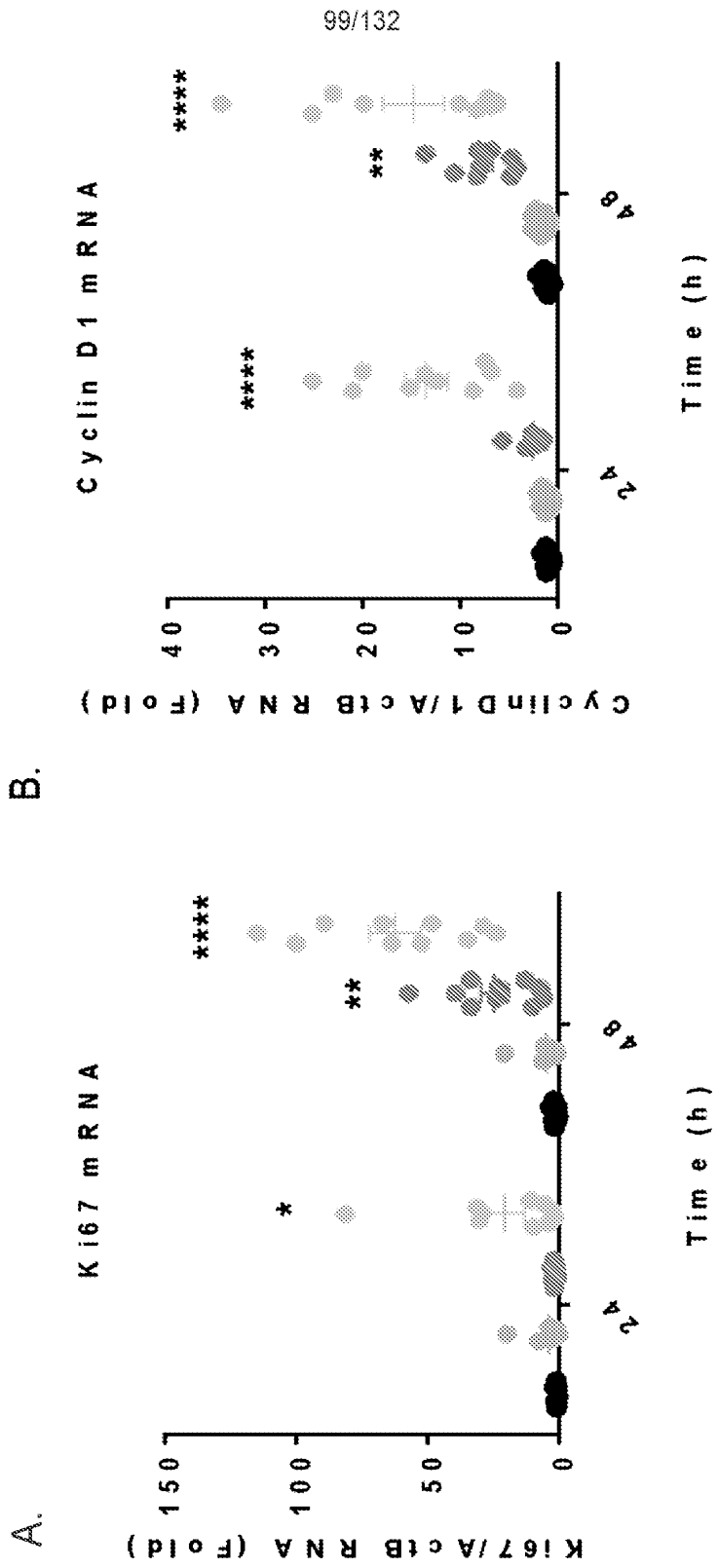

A.
Ki67 mRNA
Ki67/Actb RNA (Fold)
150
100
50
0
****
**
*
4
2
4
Time (h)
B.
Cyclin D1 mRNA
Cyclin D1/Actb RNA (Fold)
40
30
20
10
0
****
**
****
4
2
4
Time (h)

FIG. 39

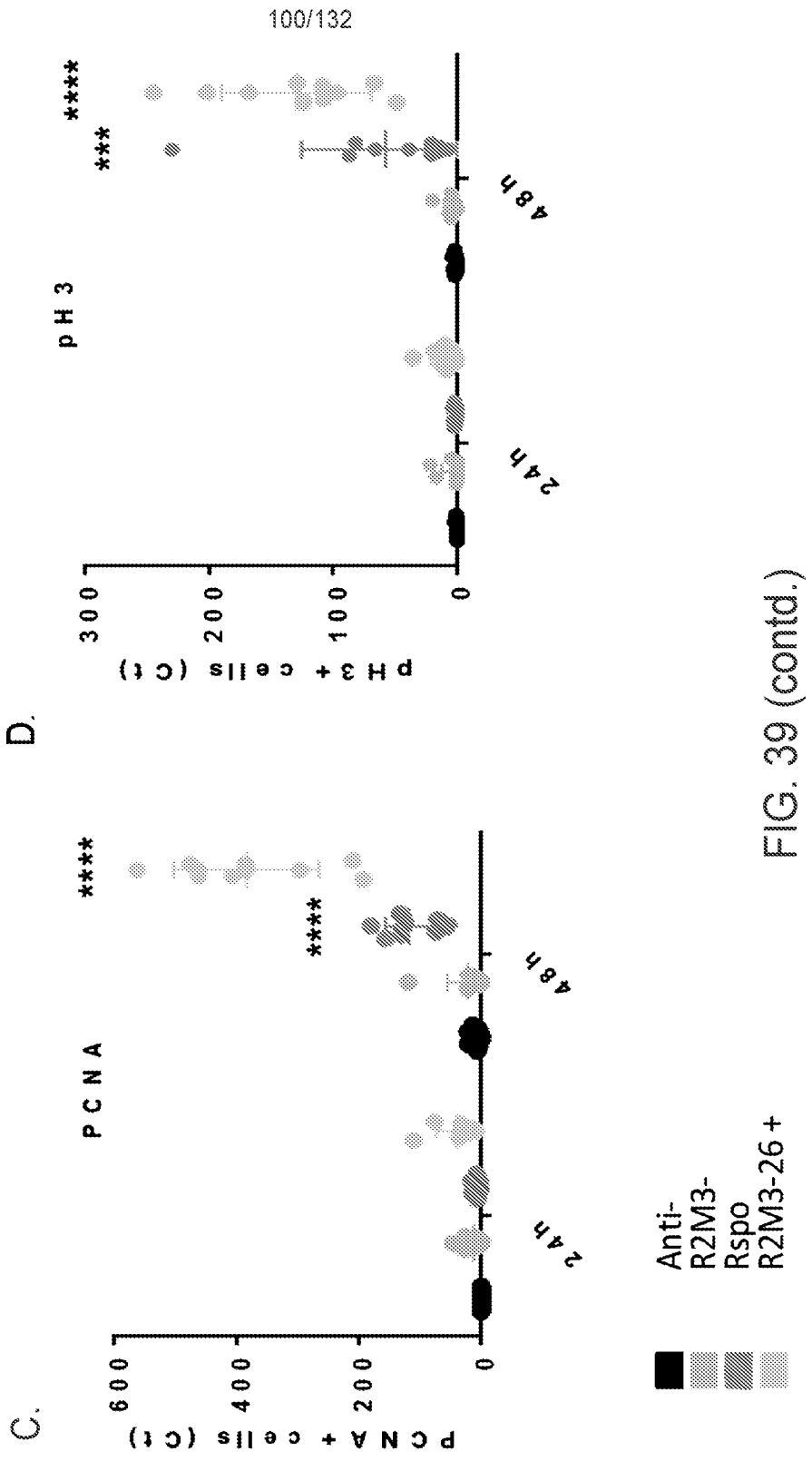
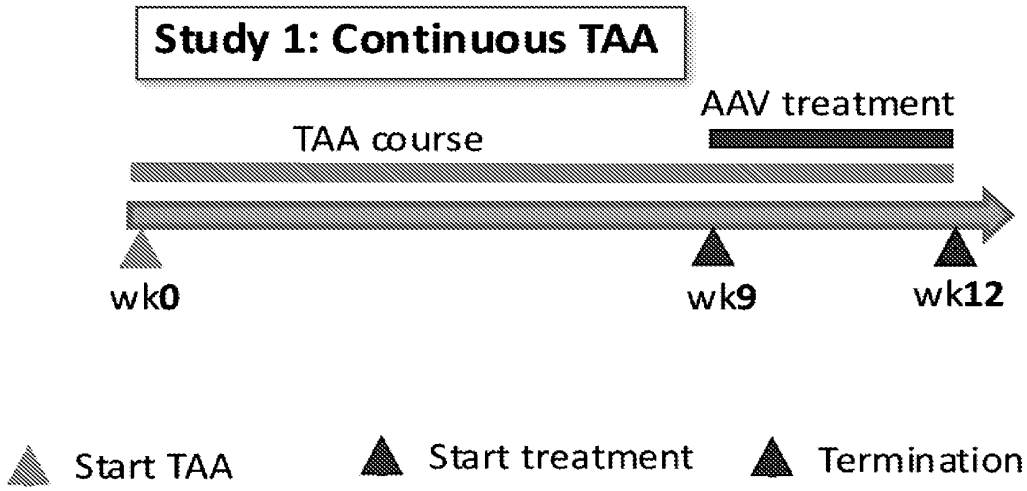

C.
PCNA
PCNA + cells (Ct)
600
400
200
0
****
****
48h
24h
D.
pH3
pH3+ cells (Ct)
300
200
100
0
****
***
48h
24h
Anti-R2M3-Rspo
R2M3-26+

FIG. 39 (contd.)

FIG. 40A

A.



B.

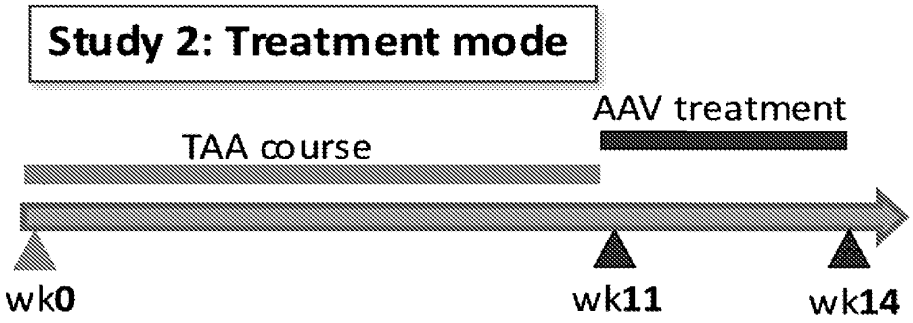


FIG. 40B

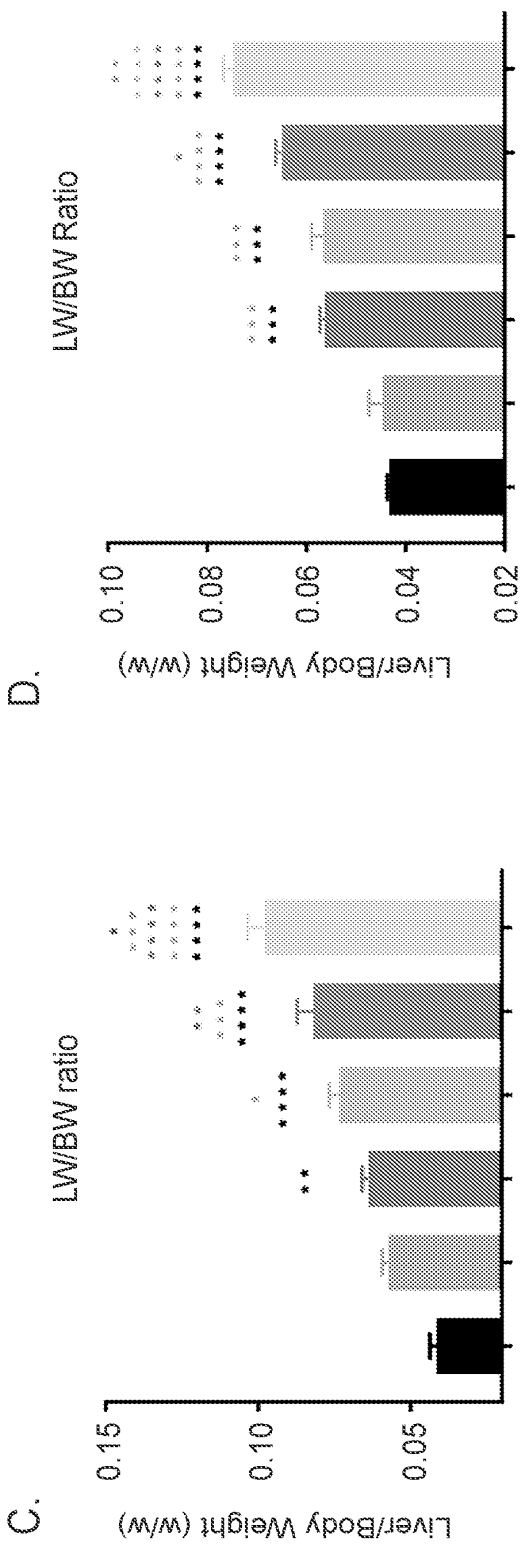
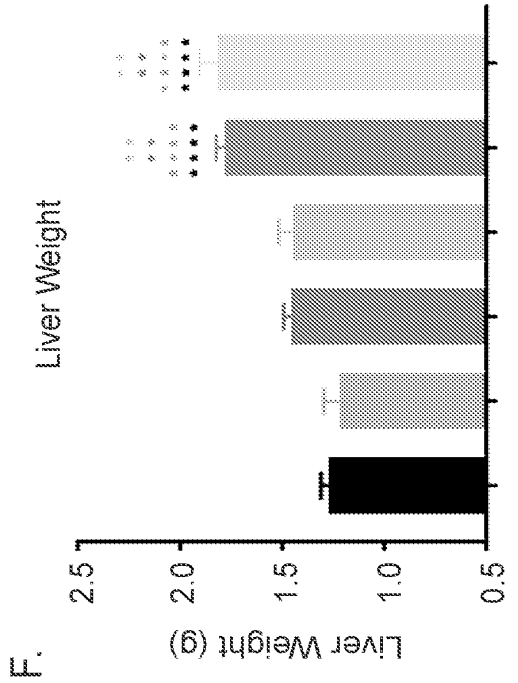

C.
LW/BW ratio
Liver/Body Weight (w/w)
0.15
0.10
0.05
D.
LW/BW Ratio
Liver/Body Weight (w/w)
0.10
0.08
0.06
0.04
0.02

FIG. 40 (contd.)


F.
Liver Weight
Liver Weight (g)
2.5
2.0
1.5
1.0
0.5

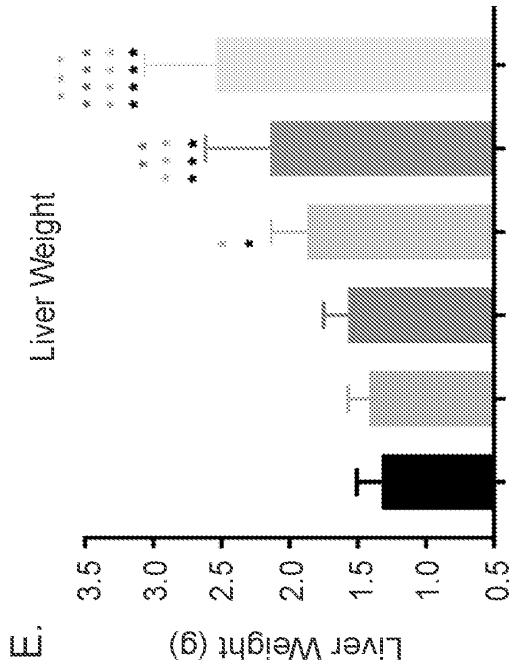

E.
Liver Weight
Liver Weight (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5

FIG. 40 (contd.)

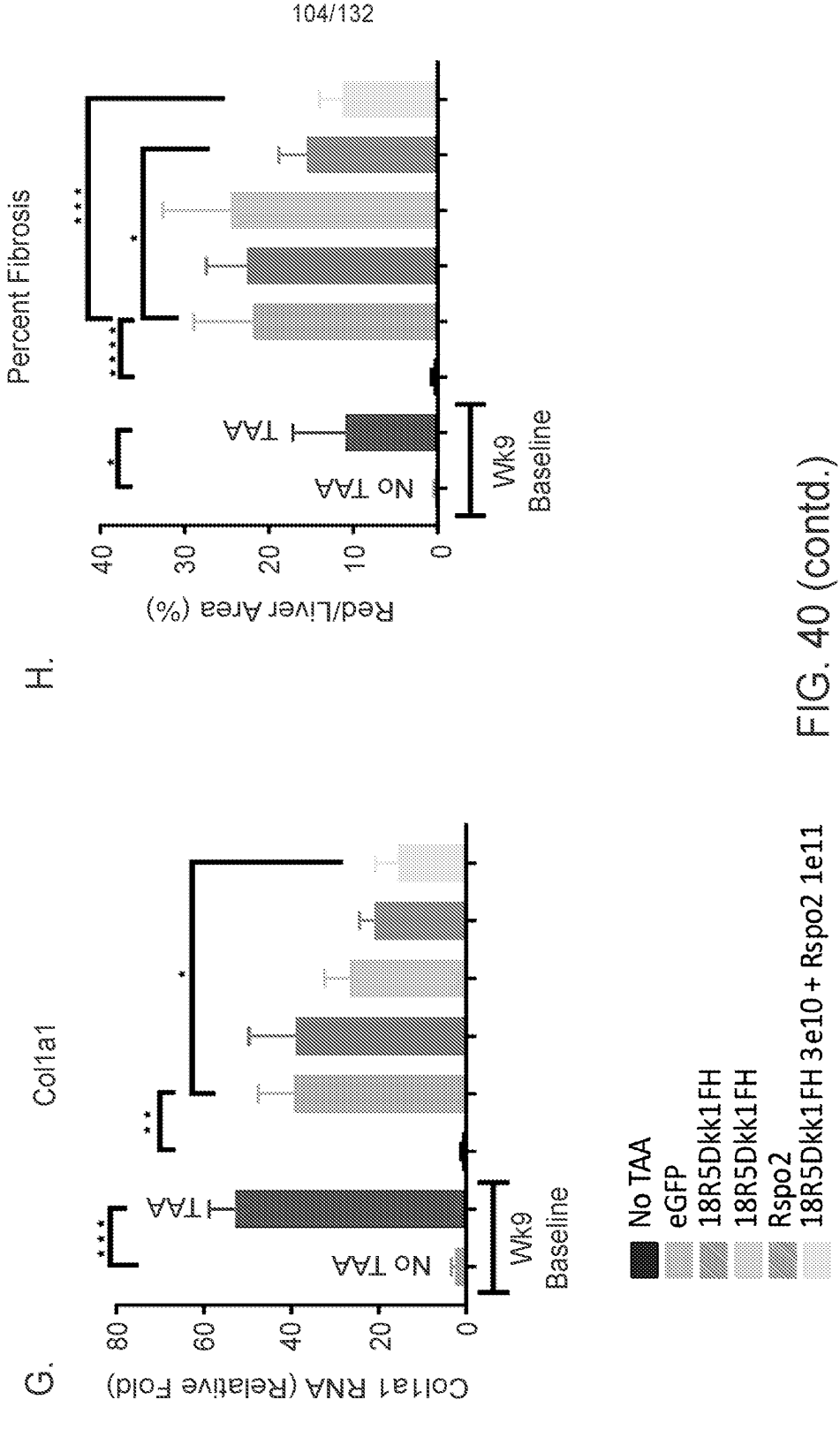
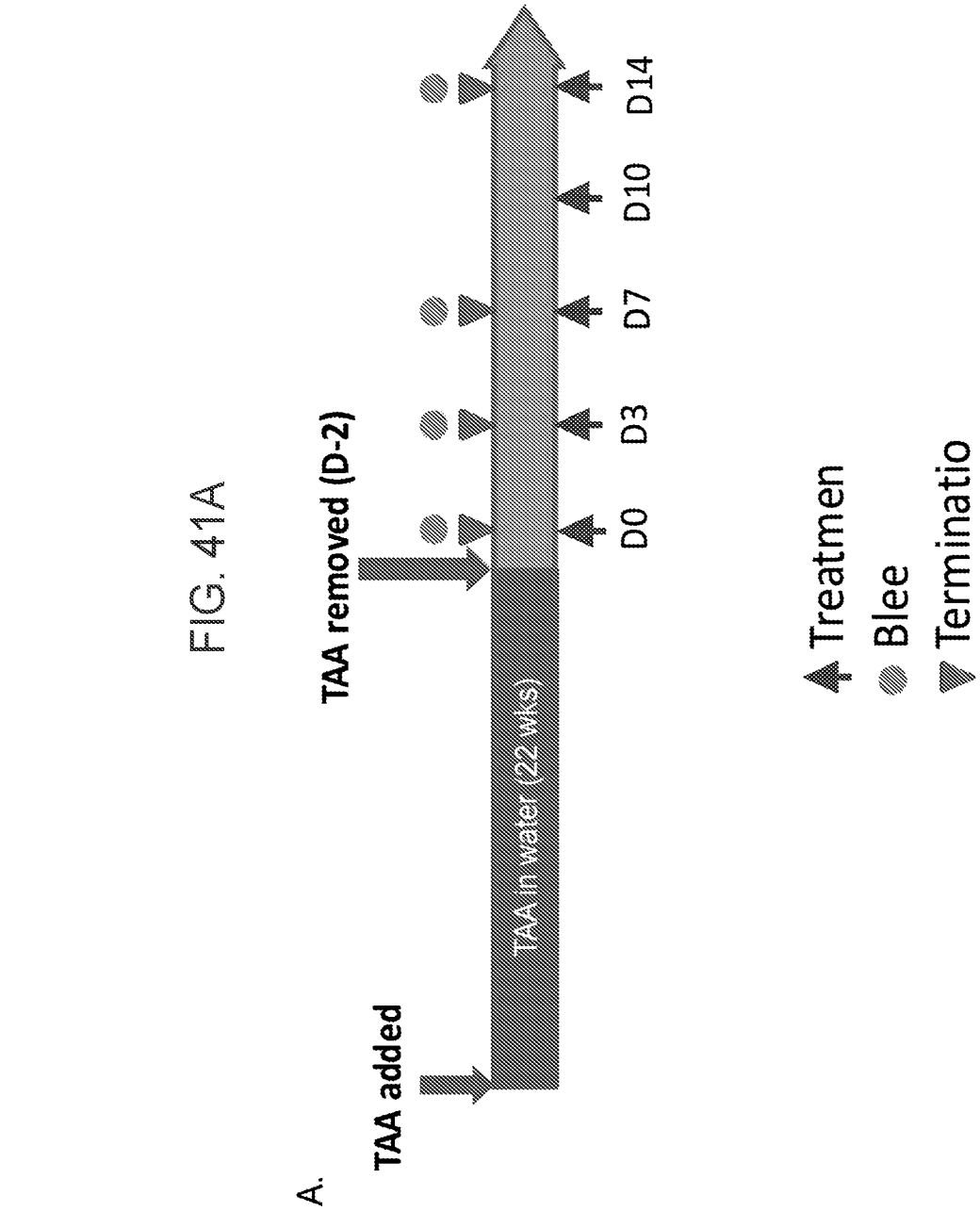

G.
Col1a1
Col1a1 RNA (Relative Fold)
0
20
40
60
80
No TAA
TAA
Wk9
Baseline
*
**
***
H.
Percent Fibrosis
Red/Liver Area (%)
0
10
20
30
40
No TAA
TAA
Wk9
Baseline
*
*
***
****
No TAA
eGFP
18R5Dkk1FH
18R5Dkk1FH
Rspo2
18R5Dkk1FH 3e10 + Rspo2 1e11

FIG. 40 (contd.)

FIG. 41A


A.
TAA added
TAA removed (D-2)
TAA in water (22 wks)
D0
D3
D7
D10
D14
Treatmen
Blee
Terminatio

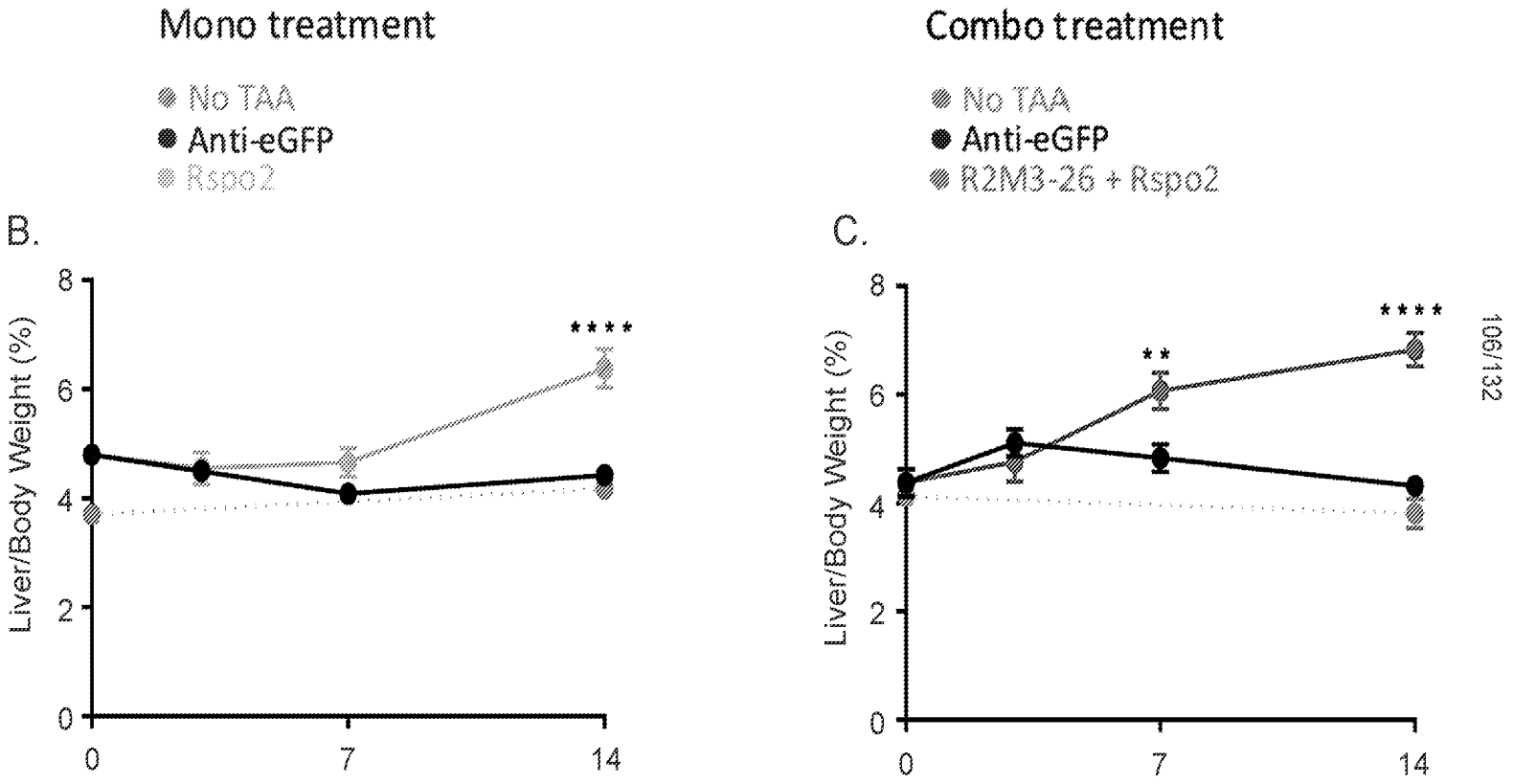


FIG. 41 (contd.)

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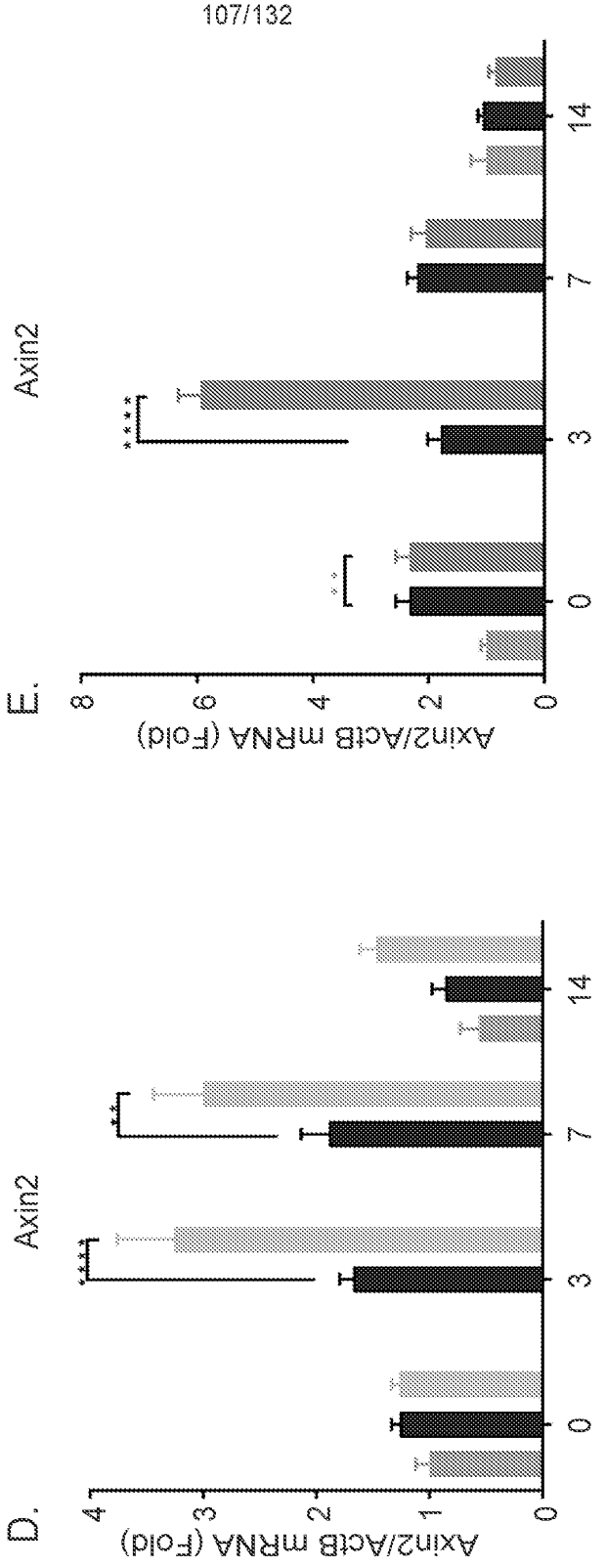

D.
Axin2
Axin2/Actβ mRNA (Fold)
0
1
2
3
4
0
3
7
14
E.
Axin2
Axin2/Actβ mRNA (Fold)
0
2
4
6
8
0
3
7
14

FIG. 41 (contd.)

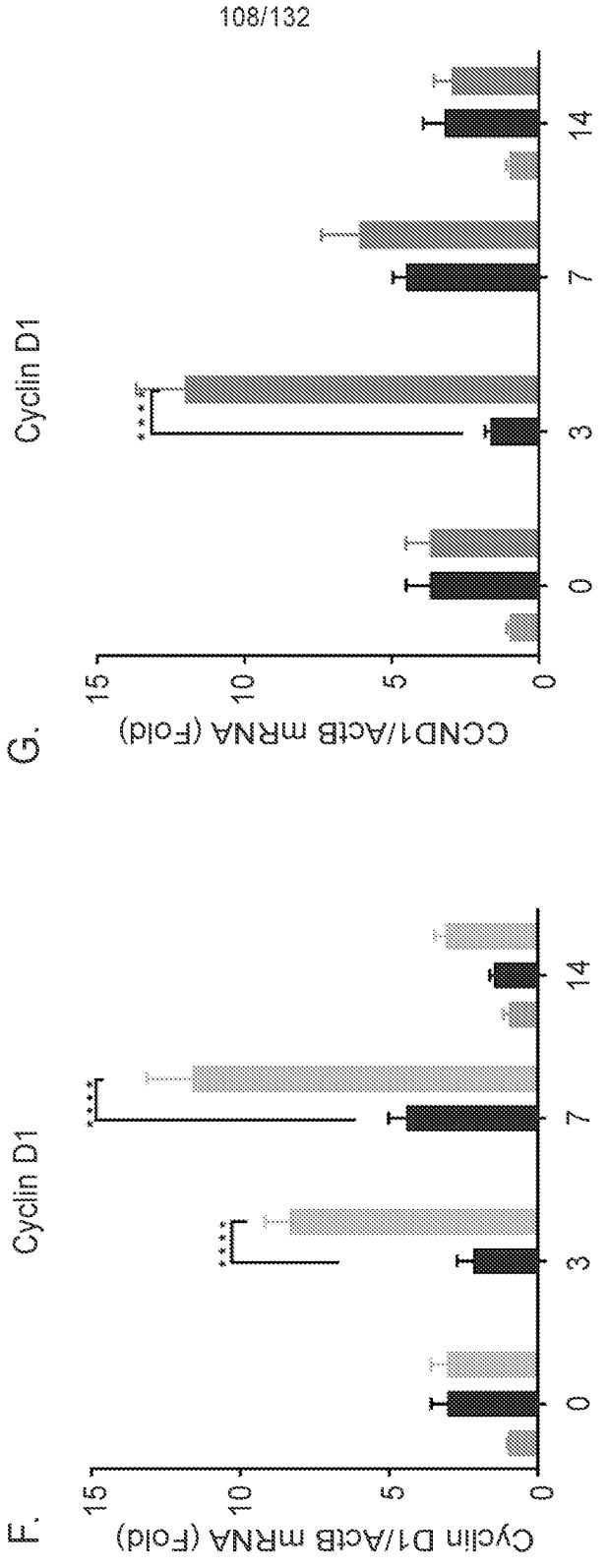

F.
Cyclin D1
Cyclin D1/ActB mRNA (Fold)
15
10
5
0
0
3
7
14
****
****
G.
Cyclin D1
CCND1/ActB mRNA (Fold)
15
10
5
0
0
3
7
14
****
****

FIG. 41 (contd.)

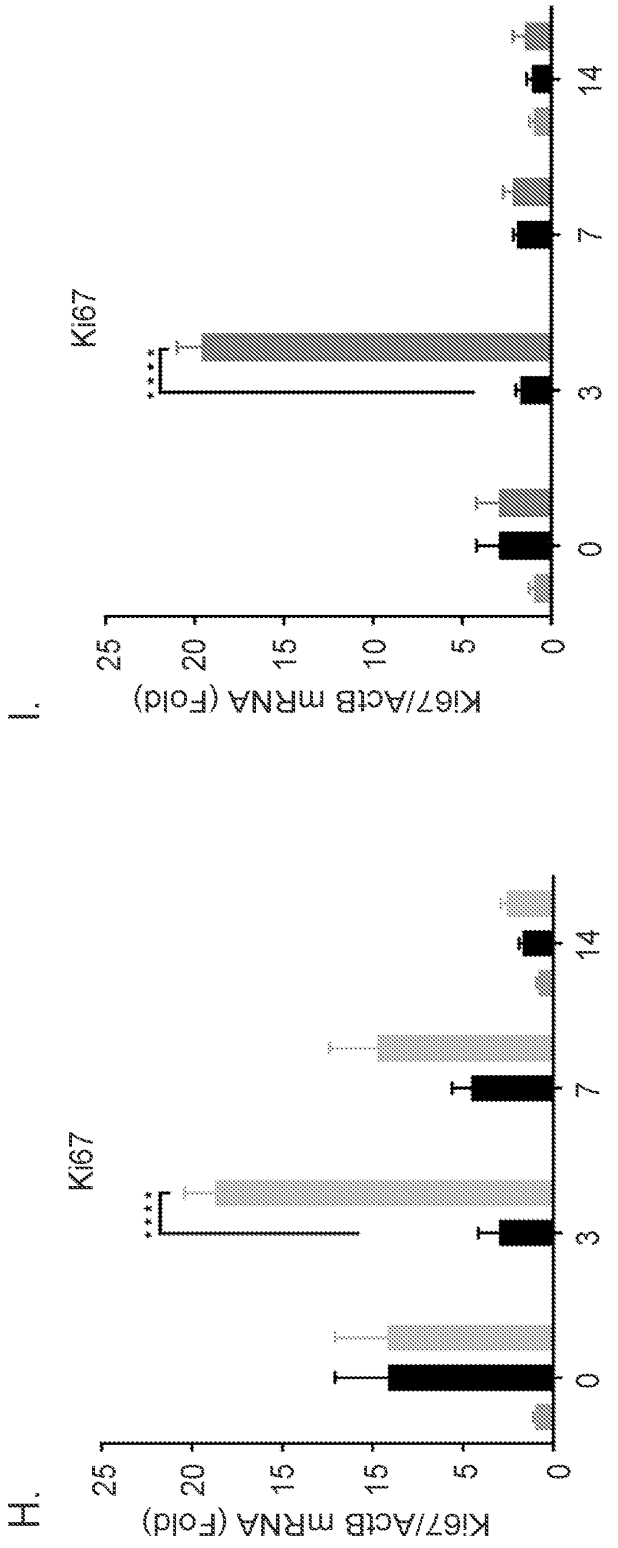

H.
Ki67
Ki67/ActB mRNA (Fold)
25
20
15
5
0
0
3
7
14
****
I.
Ki67
Ki67/ActB mRNA (Fold)
25
20
15
10
5
0
0
3
7
14
****

FIG. 41 (contd.)

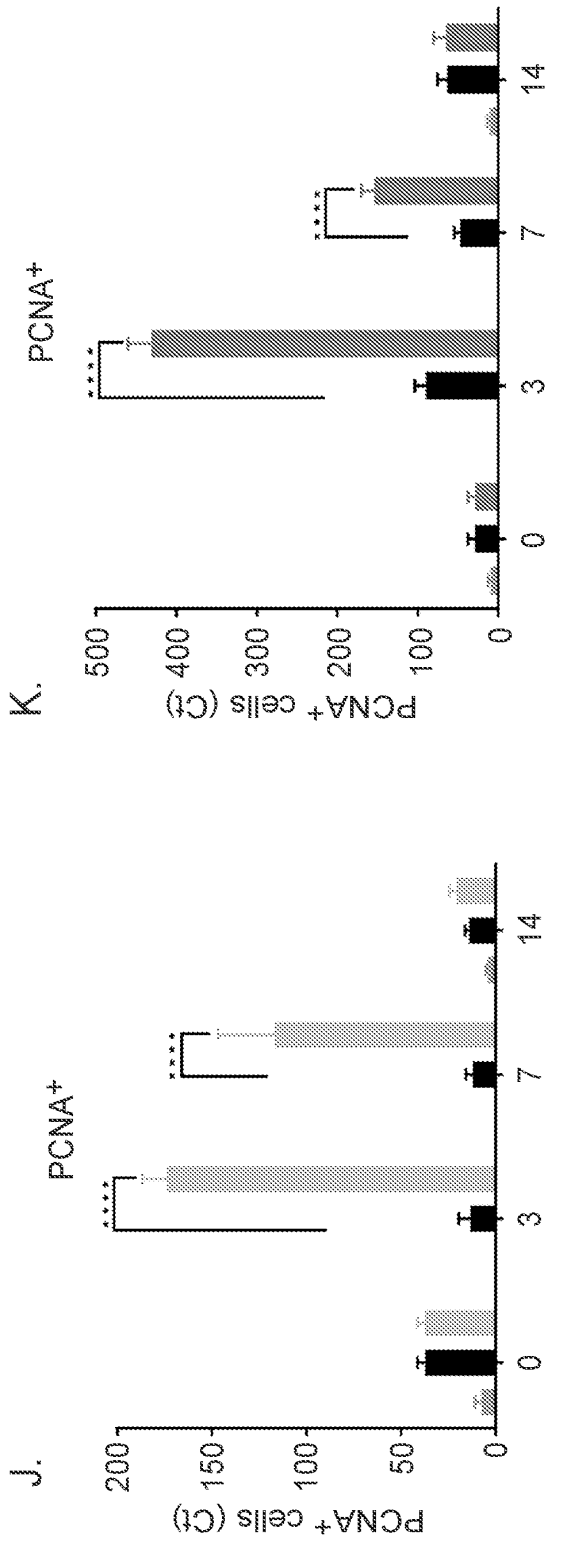

J.
PCNA+ cells (Ct)
200
150
100
50
0
PCNA+
****
****
0
3
7
14
K.
PCNA+ cells (Ct)
500
400
300
200
100
0
PCNA+
****
***
0
3
7
14

FIG. 41 (contd.)

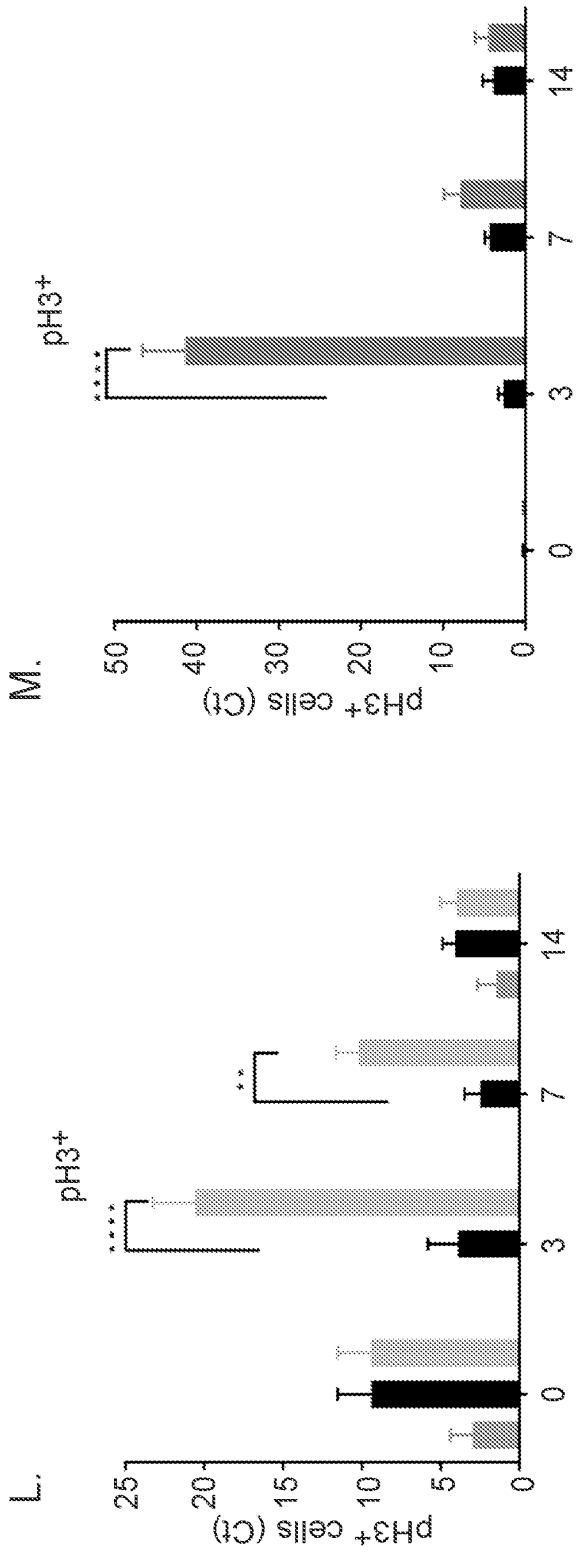

L.
pH3+ cells (Ct)
25
20
15
10
5
0
pH3+
****
**
0
3
7
14
M.
pH3+ cells (Ct)
50
40
30
20
10
0
pH3+
****
0
3
7
14

FIG. 41 (contd.)

FIG. 41N

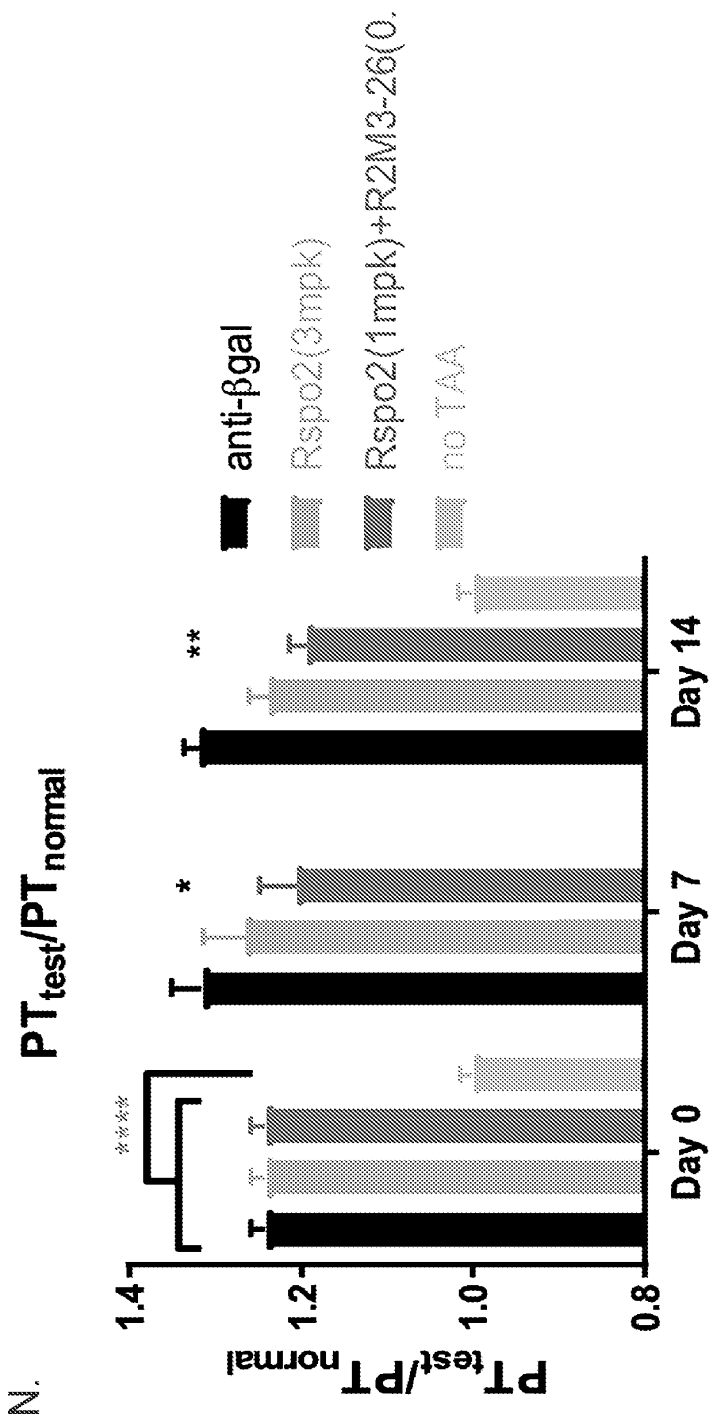

N.
PT test/PT nomal
PT test/PT normal
1.4
1.2
1.0
0.8
****
*
**
Day 0
Day 7
Day 14
anti-βgal
Rspo2(3mpk)
Rspo2(1mpk)+R2M3-26(0.
no TAA

FIG. 42

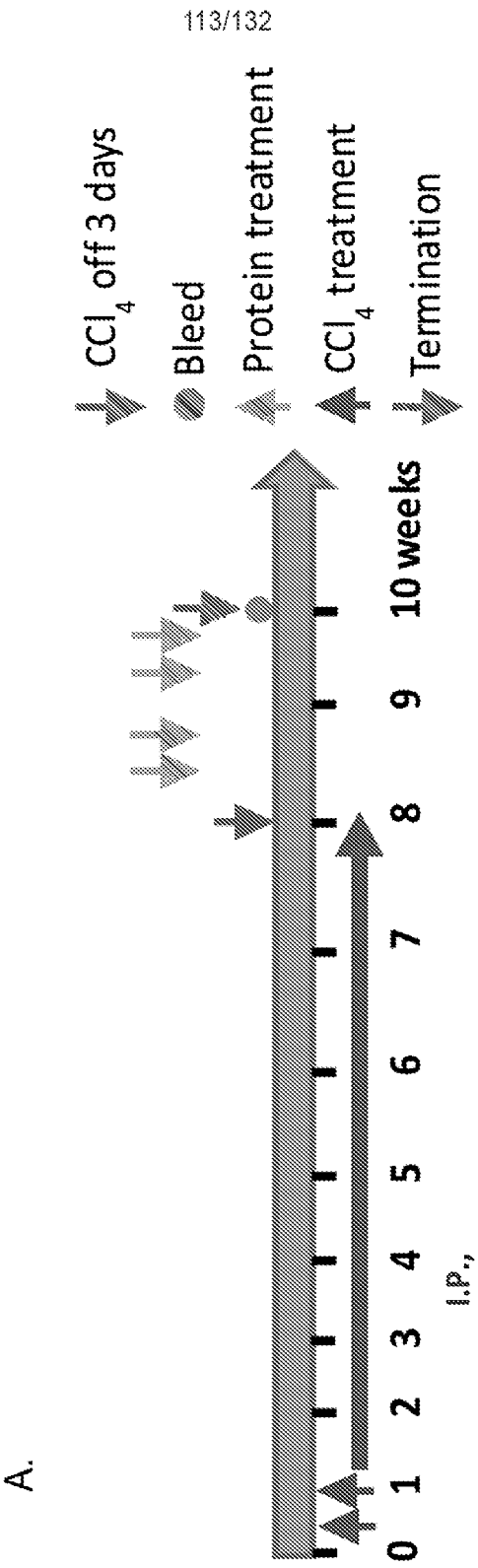

A.
0 1 2 3 4 5 6 7 8 9 10 weeks
I.P.,
CCl4 off 3 days
Bleed
Protein treatment
CCl4 treatment
Termination

FIG. 42 (contd.)

B.

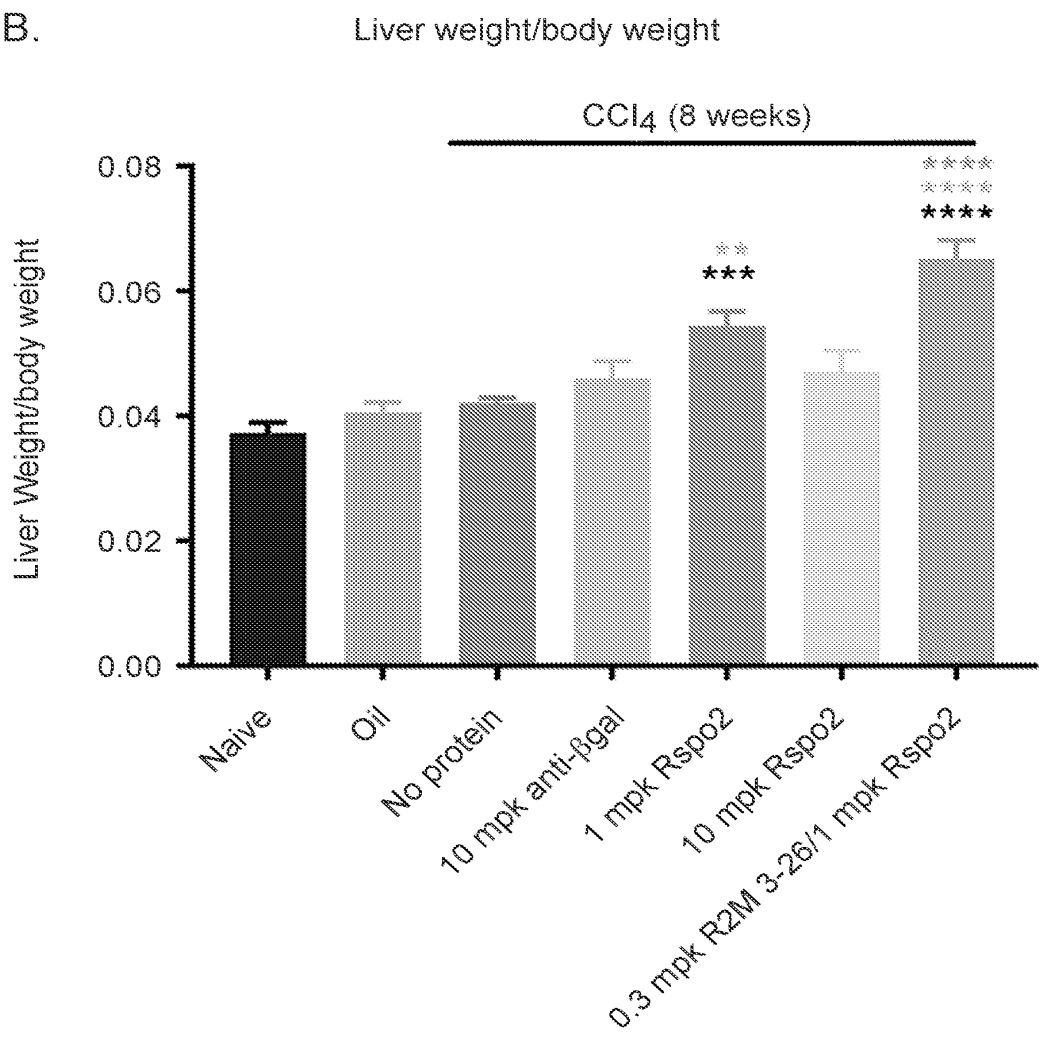


FIG. 42 (contd.)

C.

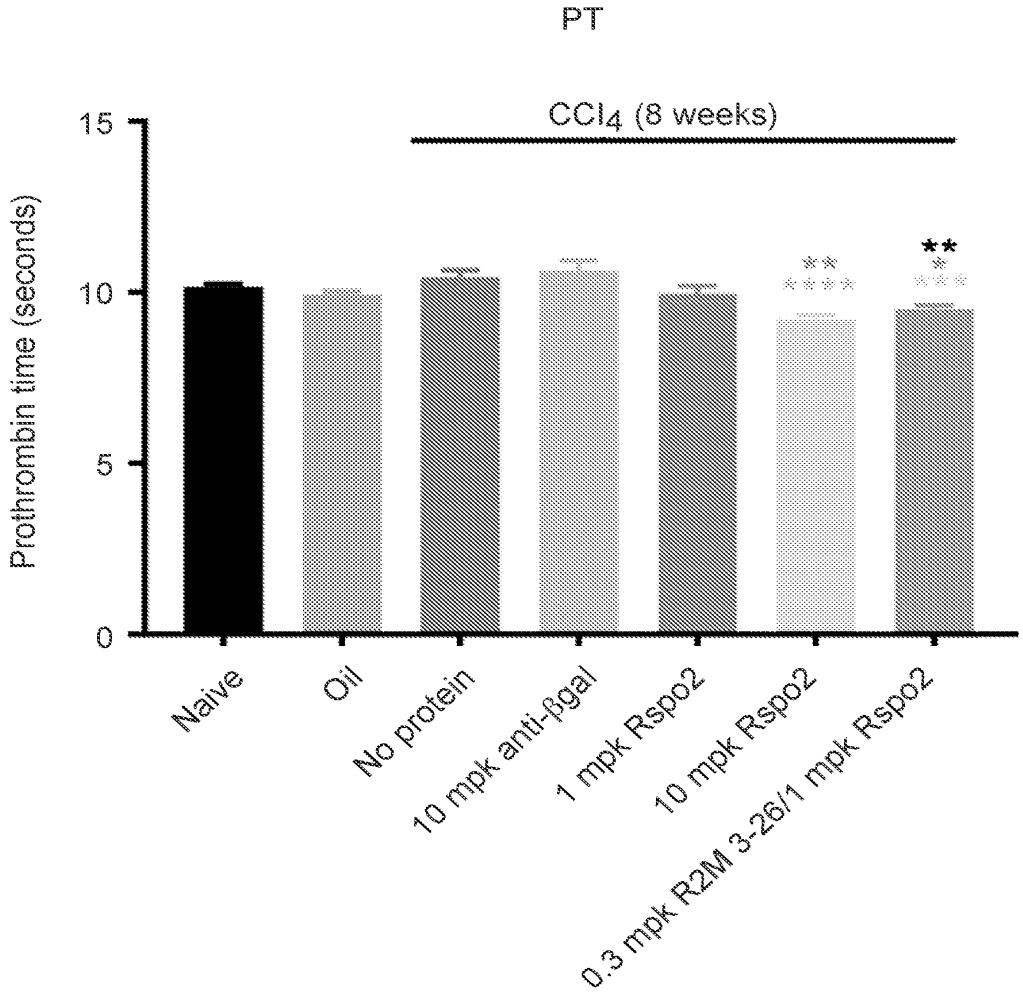


FIG. 42 (contd.)

D.

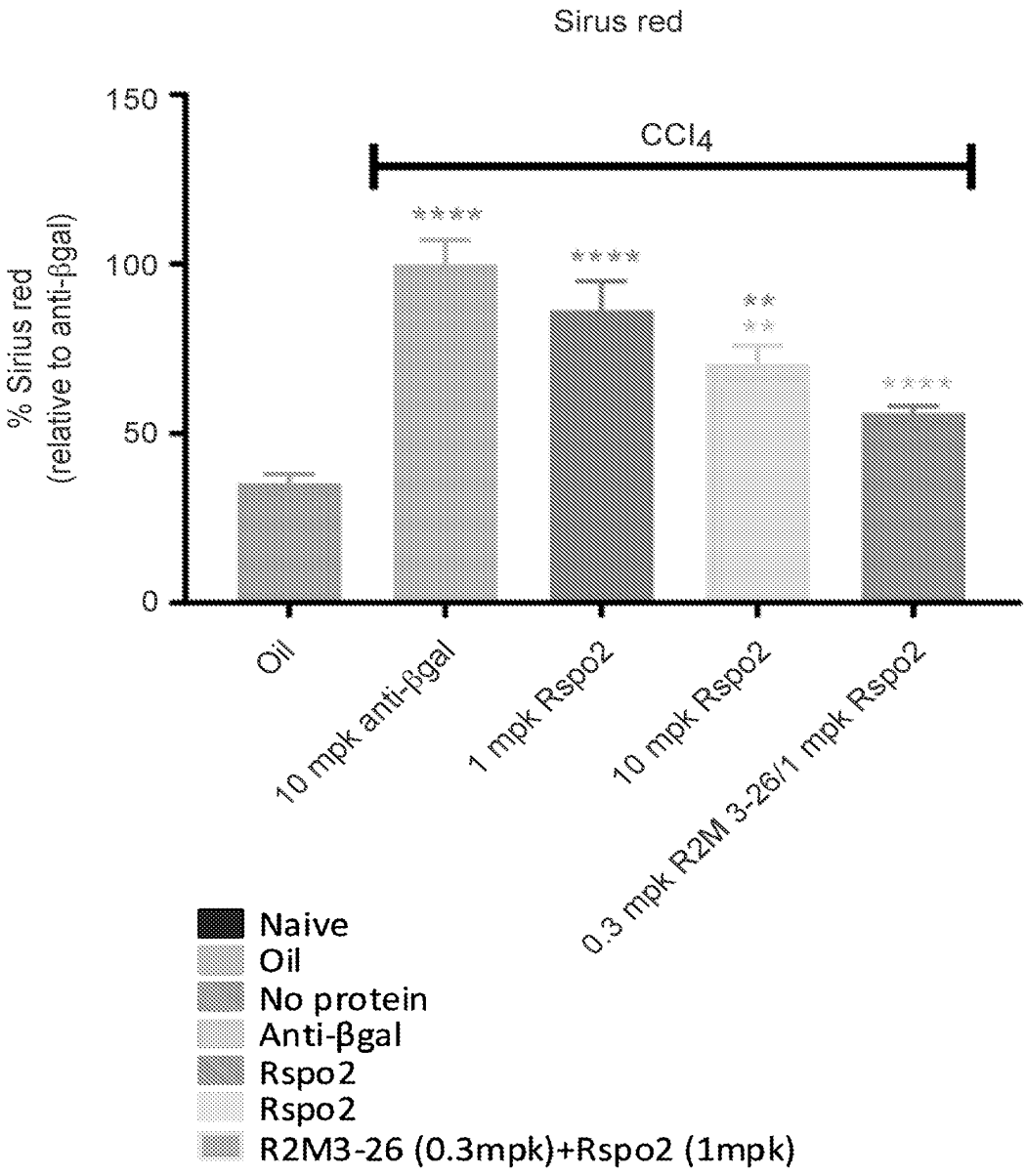


FIG. 43

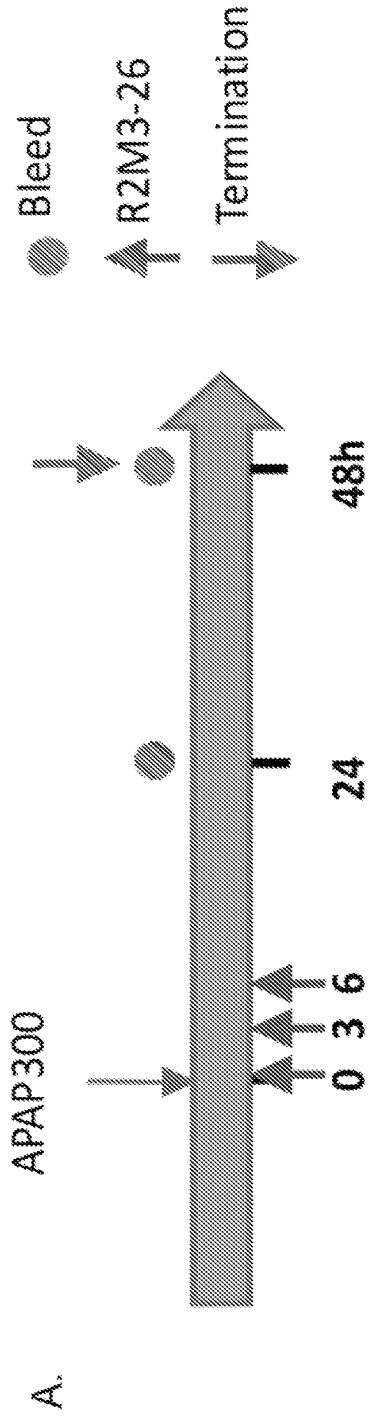
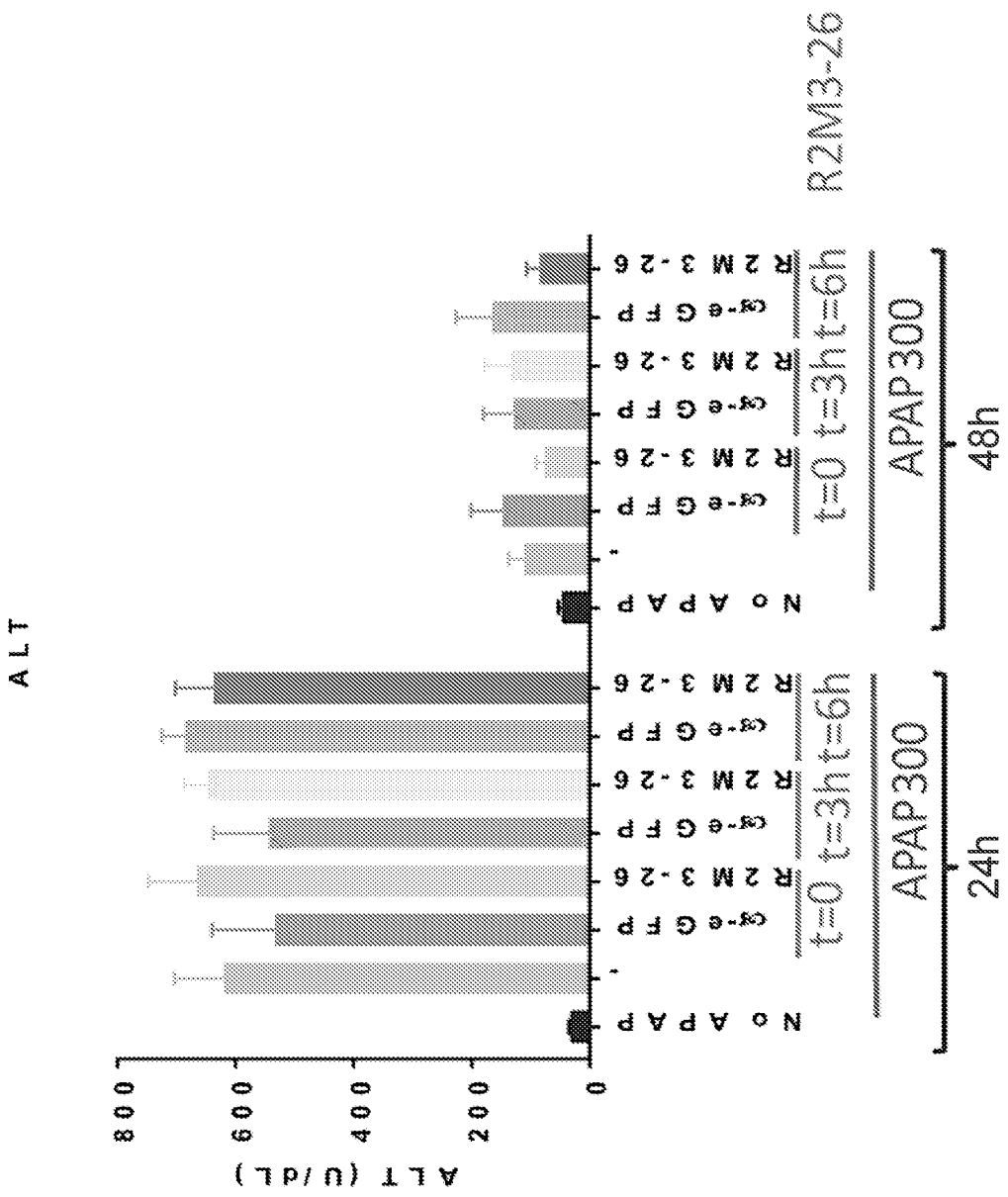


FIG. 43 (contd.)


B.
ALT
ALT (U/dL)
800
600
400
200
0
No APAP
-
α-e GFP
R2M3-26
t=0 t=3h t=6h
APAP300
24h
48h

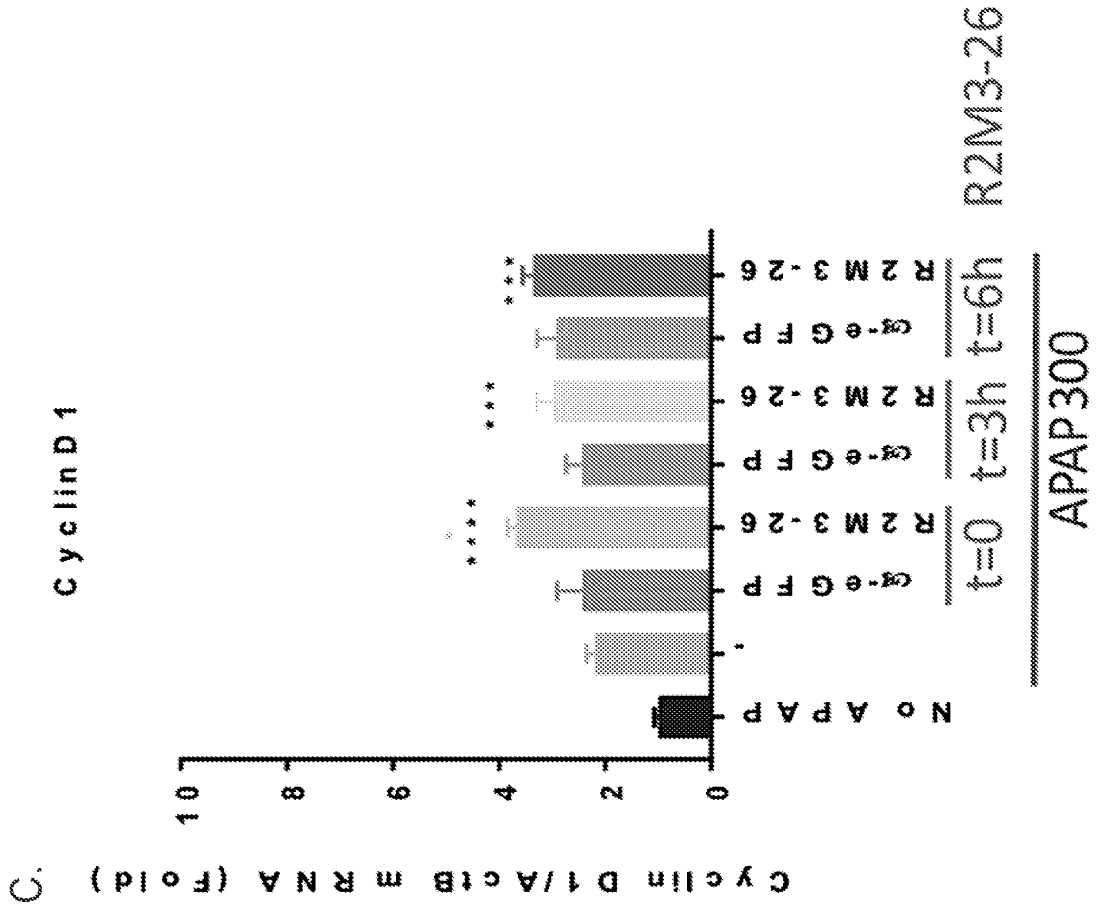

C.
Cyclin D1/Actb mRNA (Fold)
Cyclin D1
0
2
4
6
8
10
No APAP
-
α-eGFP
R2M3-26
α-eGFP
R2M3-26
α-eGFP
R2M3-26
t=0 t=3h t=6h
APAP300

FIG. 43 (contd.)

FIG. 43 (contd.)

D.

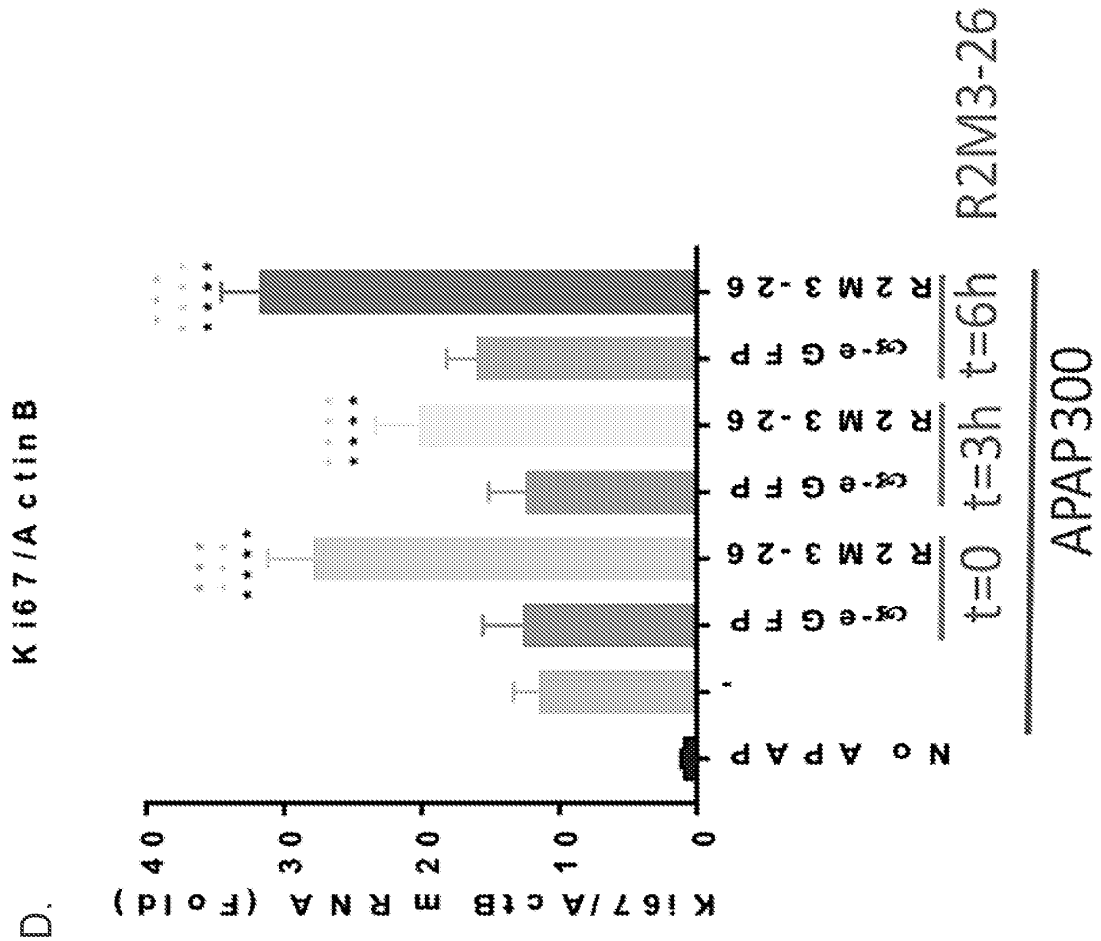


FIG. 44

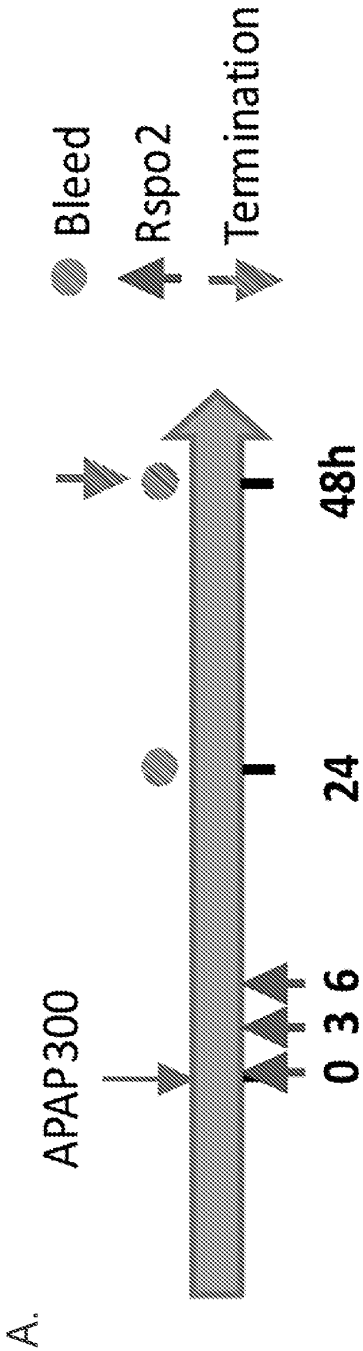
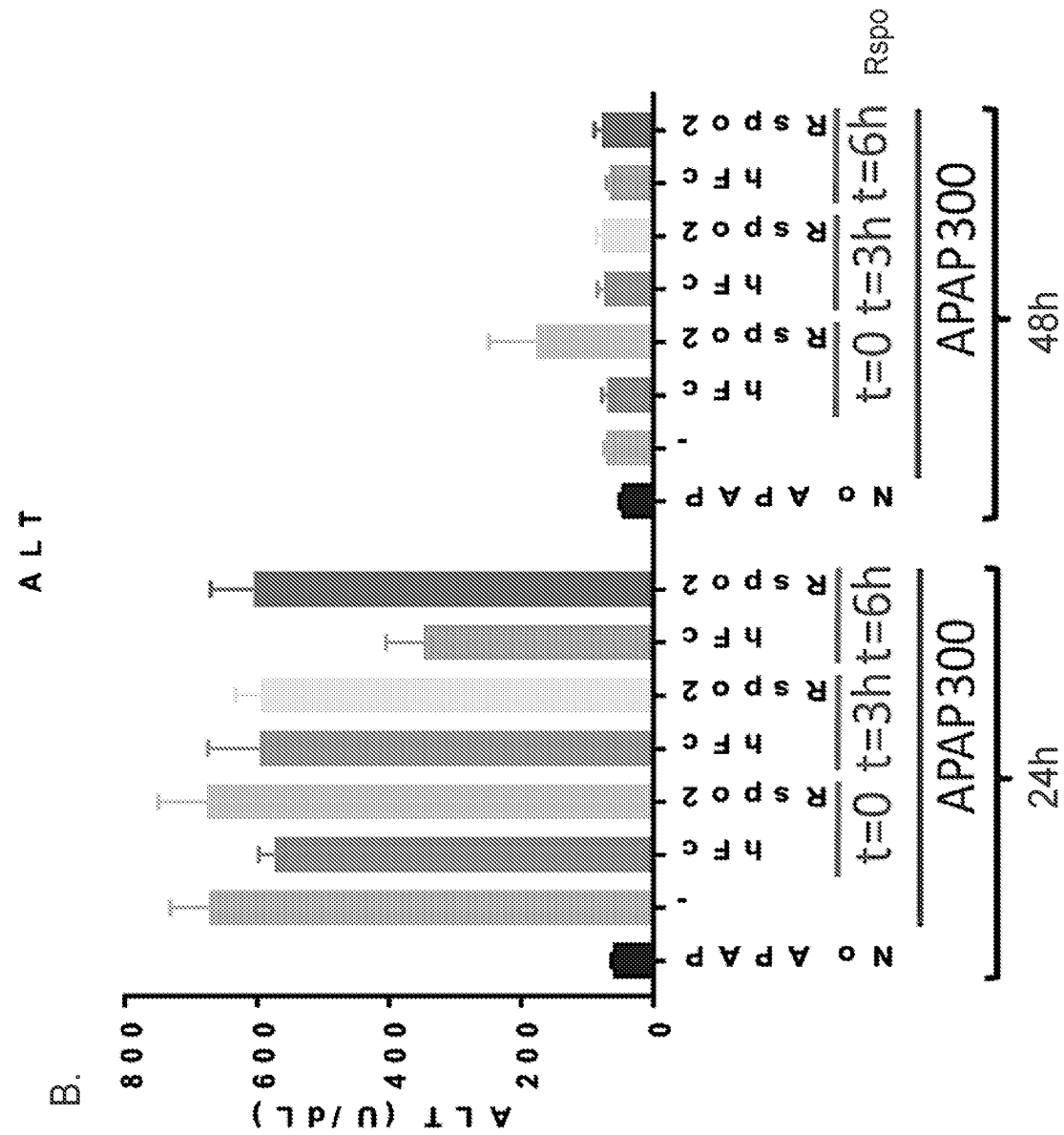


FIG. 44 (contd.) B.



C.

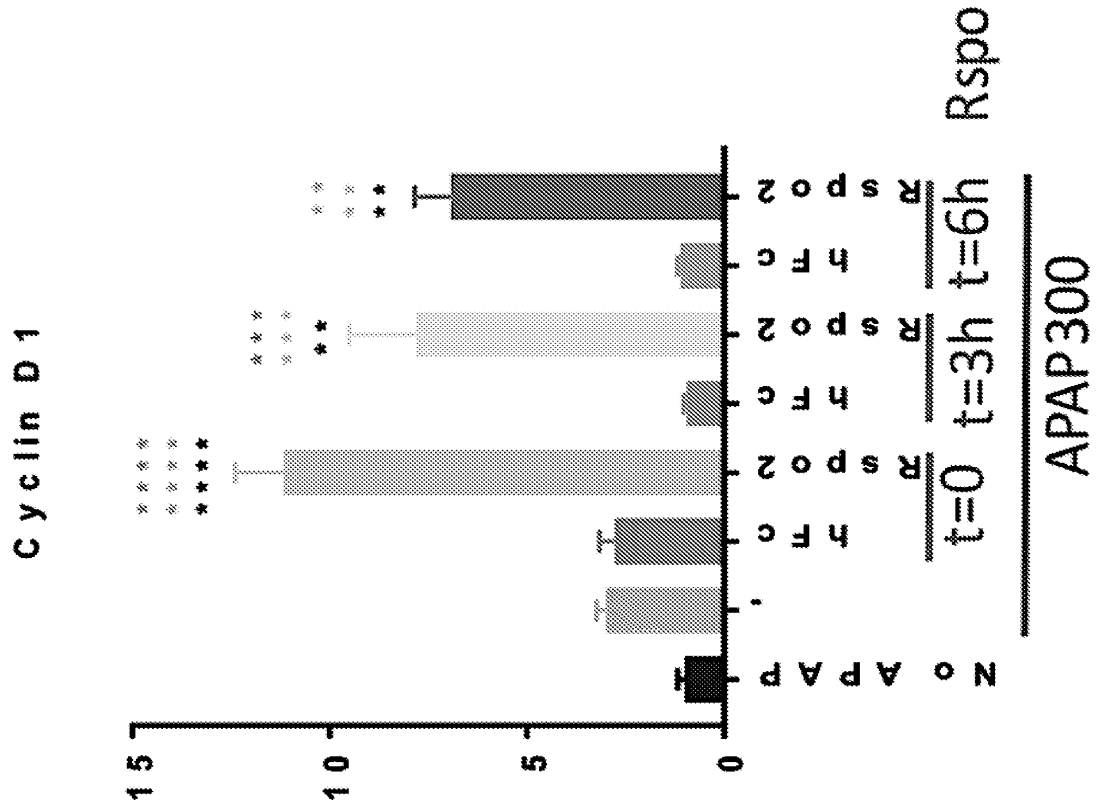


FIG. 44 (contd.)

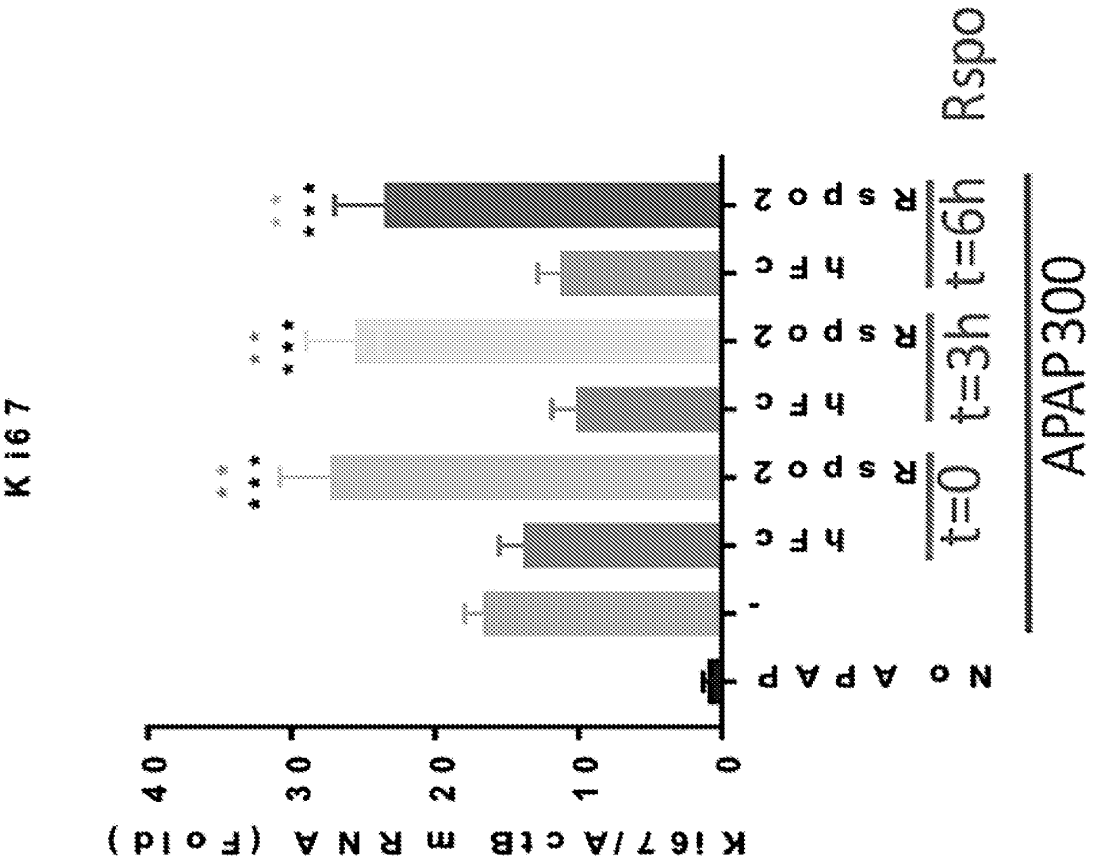


FIG. 44 (contd.) D.

FIG. 45

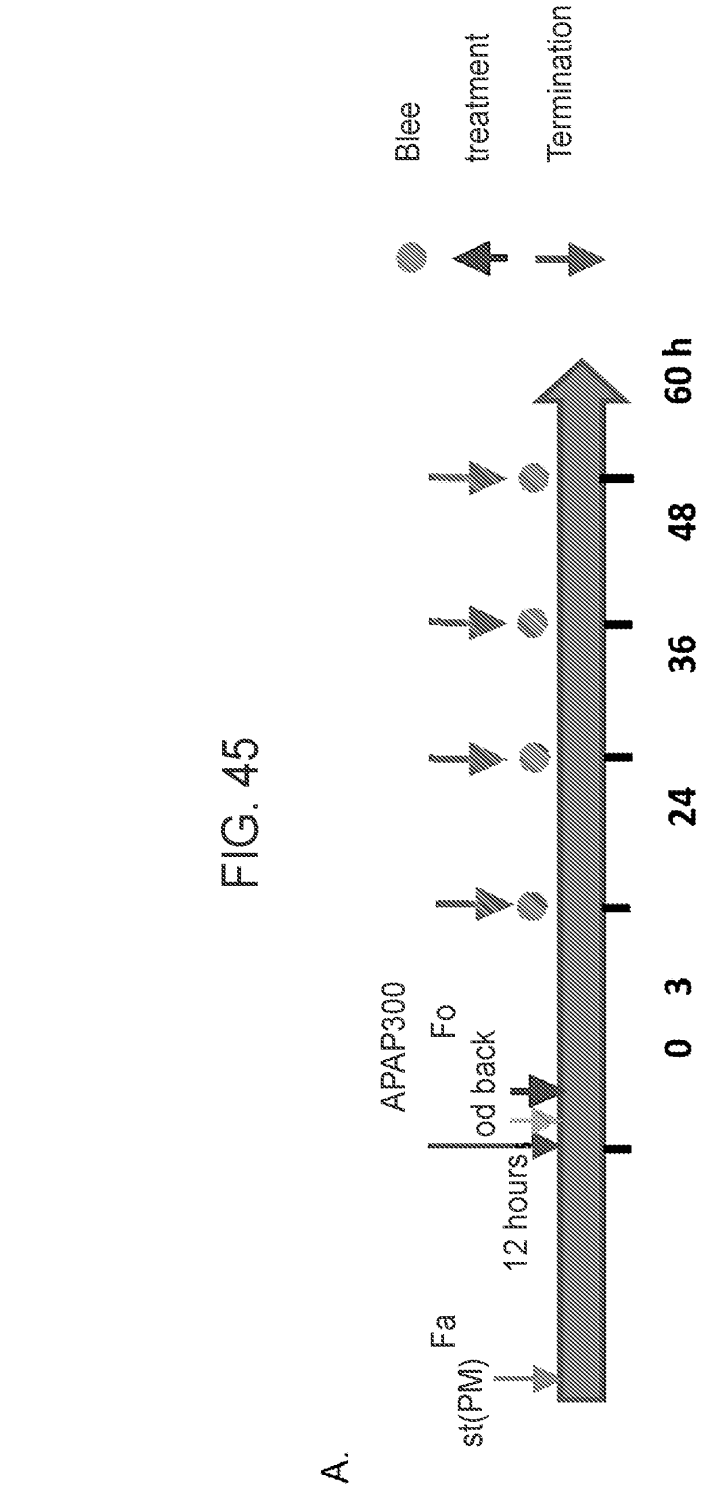
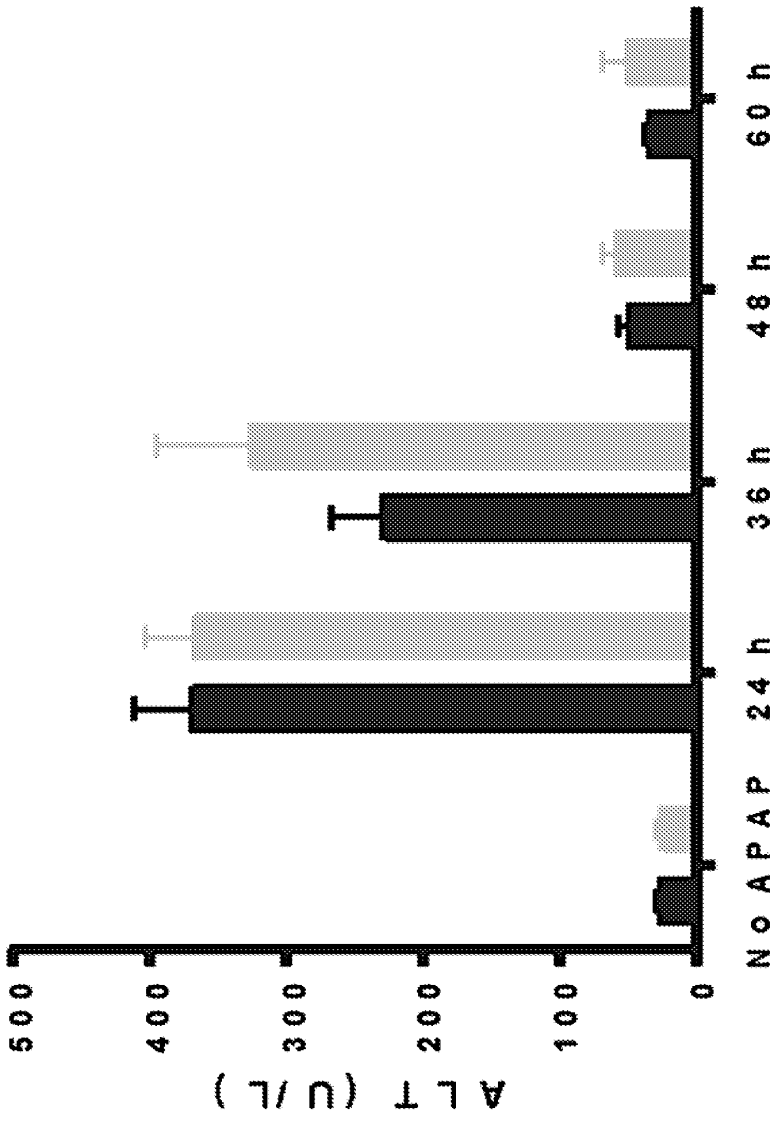
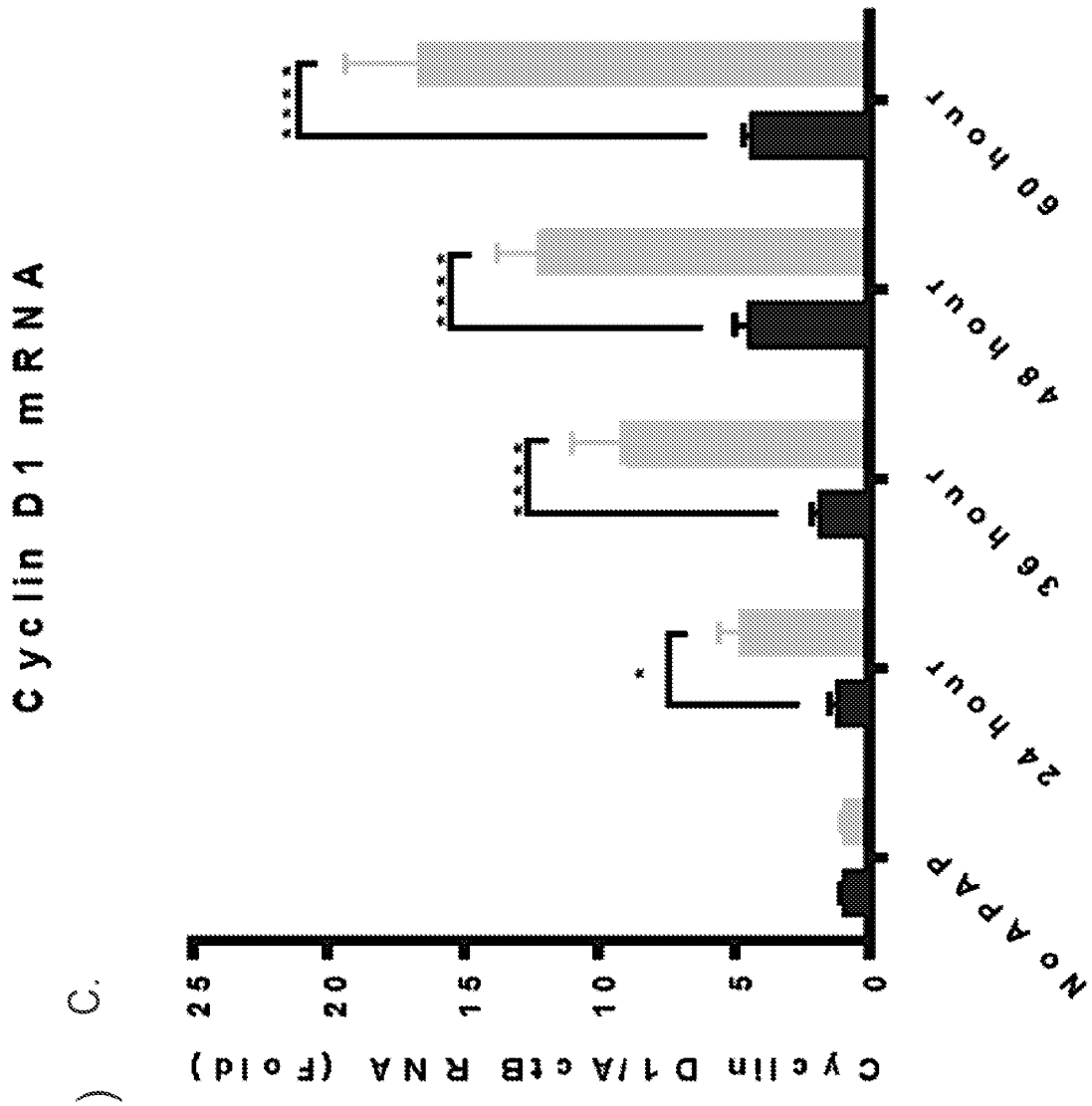

A.
Fa
st(PM)
12 hours
APAP300
Fo
od back
Blee
treatment
Termination
0
3
24
36
48
60 h

FIG. 45 (contd.)

B.





C.

FIG. 45 (contd.)

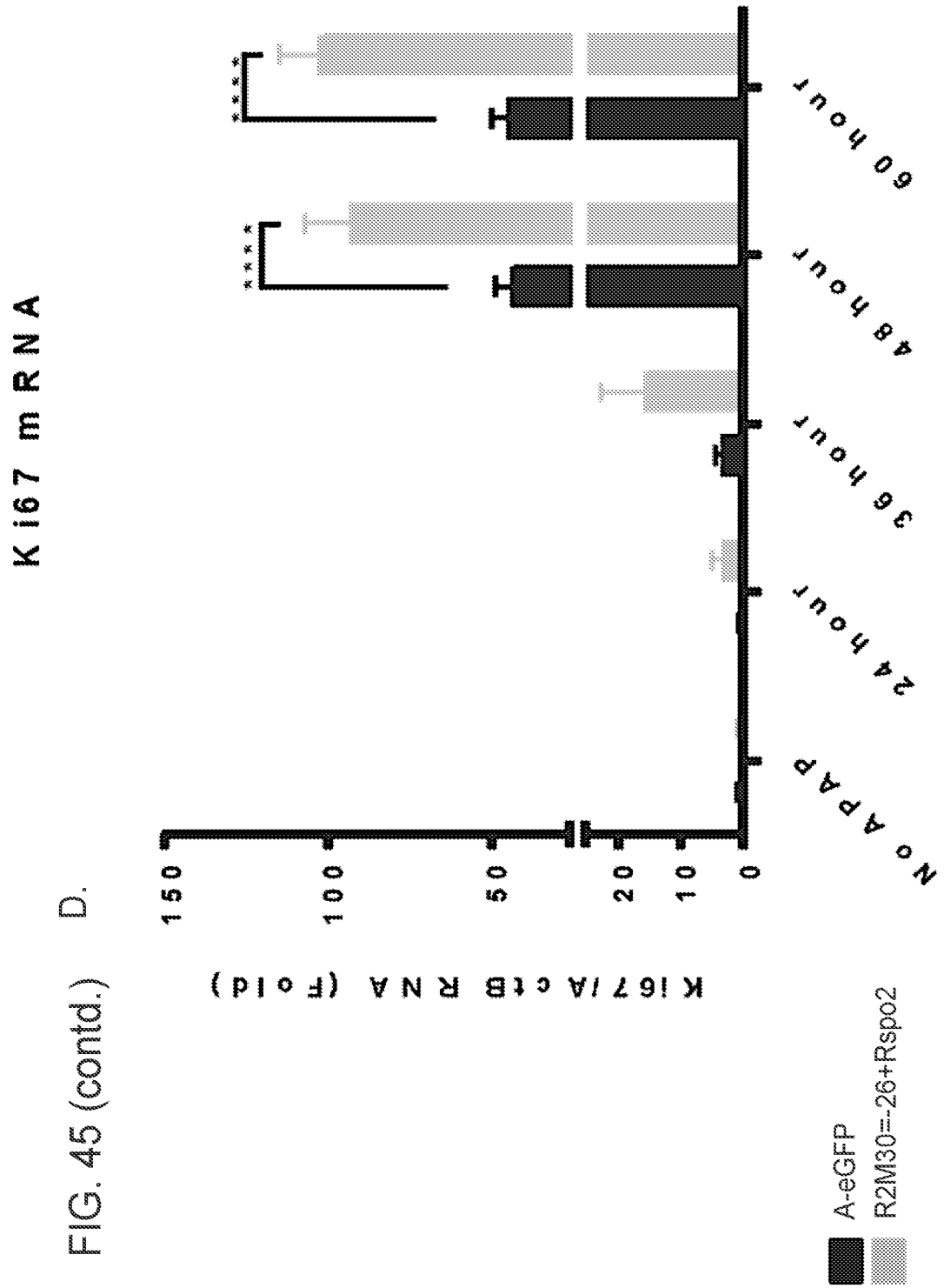
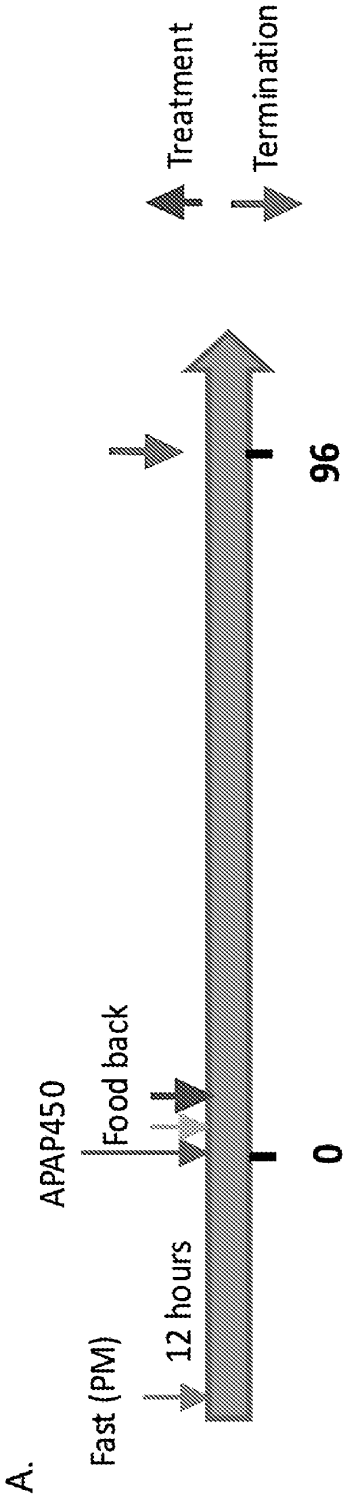

K i67 m R N A
D.
K i67/A ctB R N A (F o l d)
150
100
50
20
10
0
N o A P A P
2 4 h o u r
3 6 h o u r
4 8 h o u r
6 0 h o u r
****
****
A-eGFP
R2M30=-26+Rspo2

FIG. 45 (contd.)

FIG. 46


A.
Fast (PM)
12 hours
APAP450
Food back
0
96
Treatment
Termination

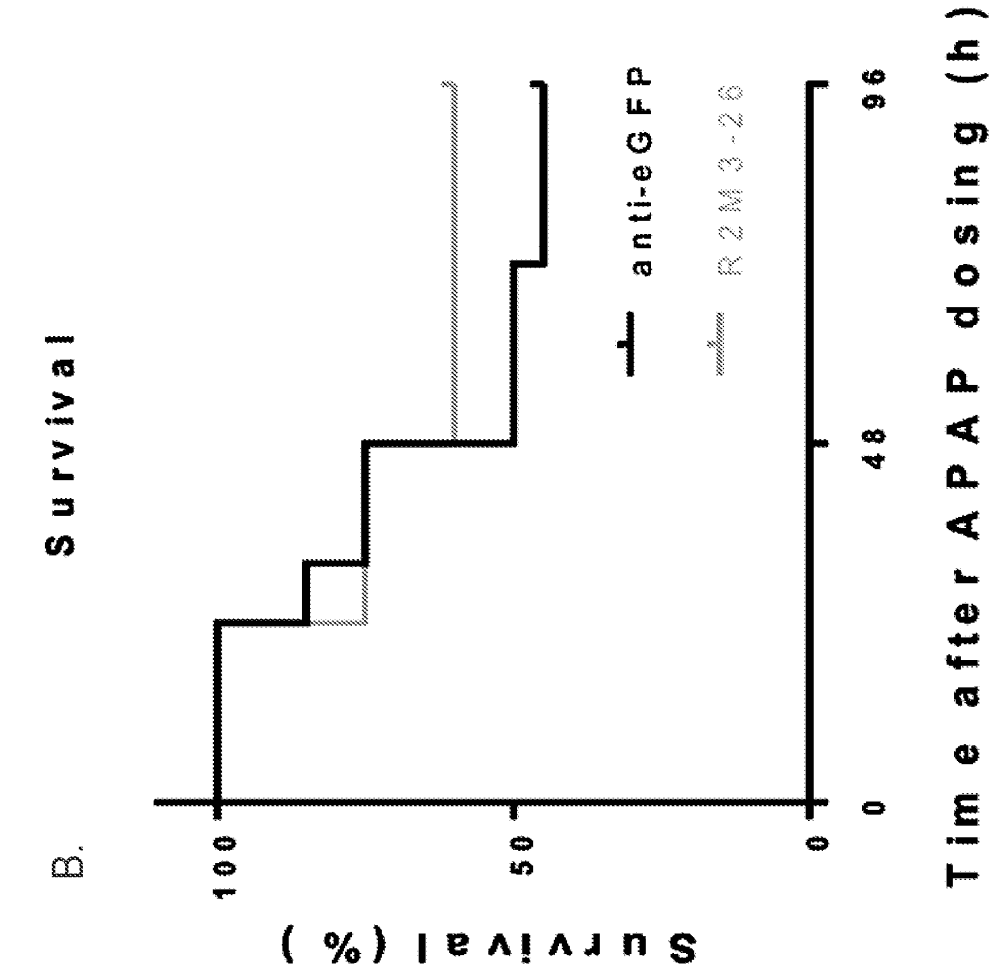

B.
Survival
Survival (%)
100
50
0
0
48
96
Time after APAP dosing (h)
anti-eGFP
R2M3-26

FIG. 46 (contd.)

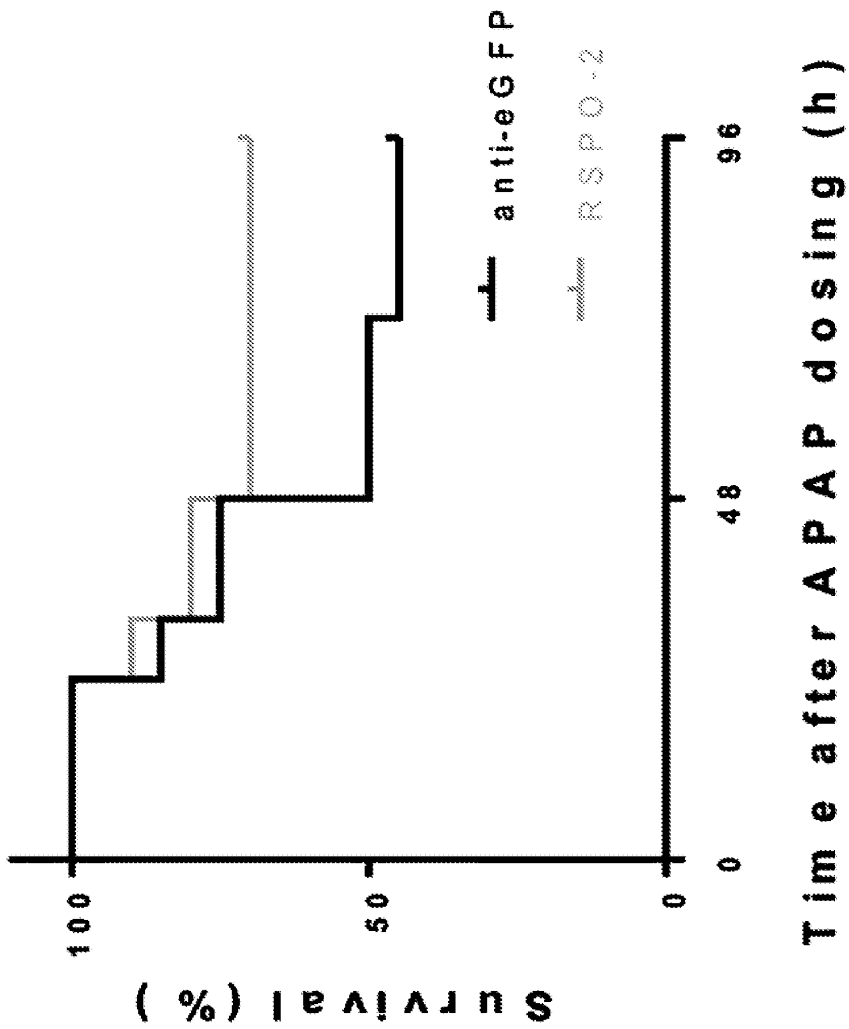
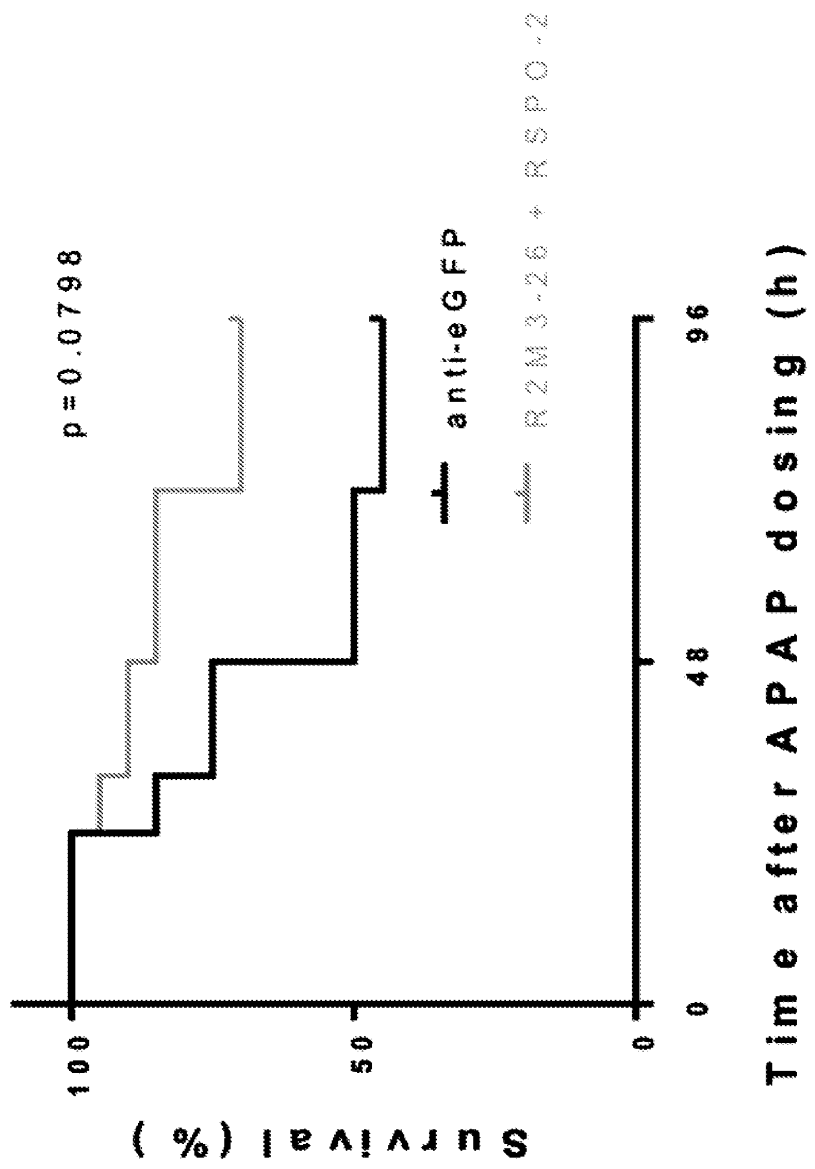

C.
Survival
Survival (%)
100
50
0
0
48
96
Time after APAP dosing (h)
anti-eGFP
RSPO-2

FIG. 46 (contd.)

FIG. 46 (contd.)

D.



Application Number: 18361782

Reviewed by: 97ClN/eHe4jhmn5GMimwpAP70alPRU2kNiDwvOWHBrv8bqgC4kdzMg==

Note: This validation report concerns the sequence listing filed 07-28-2023 21:15:59 and indicates that NO errors were found by the USPTO during validation of the sequence listing. This validation report should not be construed as meaning that the sequence listing complies with all of the requirements of 37 CFR §§ 1.821-1.825, 37 CFR §§ 1.831-1.835 and 1.839, WIPO Standard ST.25, or WIPO Standard ST.26.

For any sequence listing related questions or concerns, the Applicant is invited to contact the USPTO Sequence Listing Help Desk at 571-272-2510 or SequenceHelpDesk@USPTO.GOV.

Timestamp: [year=2023; month=7; day=31; hr=13; min=11; sec=51; ms=892]

Validated By CRFValidator v 1.5.0

Application No: 18361782 Version No: 1.1

CRF Copy Request from application Version :

Input Set: /slic/CRF_WORKFLOW/18/361/782/1.1/report_18361782_1_1.xml

Output Set: /slic/CRF_WORKFLOW/18/361/782/1.1/report_18361782_1_1.xml.pdf

Started: 2023-07-28 21:18:20.000

Finished: 2023-07-28 21:18:51.630

Elapsed: 0 hr(s) 0 min(s) 31 sec(s) 630 ms

Total Warnings: 0

Total Errors: 0

No. of SeqIDs Defined: 2267

Actual SeqID Count: 2267

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Form used in lieu of PTO/SB/08a/b (07-09)
Approved for use through 05/31/2024. OMB 0651-0031
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 41

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Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

U. S. PATENT DOCUMENTS

Examiner Initials*		Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		001	US-4946778	08-07-1990	LADNER et al.	
		002	US-5091513	02-25-1992	HUSTON; James S. et al.	
		003	US-5132405	07-21-1992	HUSTON; James S. et al.	
		004	US-6005079	12-21-1999	CASTERMAN et al.	
		005	US-6528624	03-04-2003	IDUSOGIE; Esohe Ekinaduese et al.	
		006	US-6538124	03-25-2003	IDUSOGIE; Esohe Ekinaduese et al.	
		007	US-6765087	07-20-2004	CASTERMAN et al.	
		008	US-6838254	01-04-2005	HAMERS et al.	
		009	US-7297775	11-20-2007	IDUSOGIE; Esohe Ekinaduese et al.	
		010	US-7317091	01-08-2008	LAZAR; Gregory Alan et al.	
		011	US-7364731	04-29-2008	IDUSOGIE; Esohe Ekinaduese et al.	
		012	US-7657380	02-02-2010	LAZAR; Gregory Alan et al.	
		013	US-7662925	02-16-2010	LAZAR; Gregory Alan et al.	
		014	US-7982013	07-19-2011	GURNEY et al.	
		015	US-8202966	06-19-2012	McCARTHY; Sean A.	
		016	US-8221751	07-17-2012	NAKAMURA et al.	
		017	US-8343922	01-01-2013	WU et al.	
		018	US-8461155	06-11-2013	WU et al.	
		019	US-8507442	08-13-2013	GURNEY et al.	
		020	US-8637506	01-28-2014	WU et al.	
		021	US-8715941	05-06-2014	ABO et al.	
		022	US-8846041	09-30-2014	BOURHIS et al.	

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Sheet 2 of 41

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		023	US-8859736	10-14-2014	MA et al.	
		024	US-8883735	11-11-2014	JENKINS et al.	
		025	US-8975044	03-10-2015	GURNEY et al.	
		026	US-9359444	06-07-2016	DUPONT et al.	
		027	US-9573998	02-21-2017	GURNEY et al.	
		028	US-11142577	10-12-2021	GARCIA et al.	
		029	US-20030044409	03-06-2003	CARSON; Dennis A. et al.	
		030	US-20030099647	05-29-2003	DESHPANDE; Rajendra V. et al.	
		031	US-20030157109	08-21-2003	CORVALAN; Jose R. F. et al.	
		032	US-20030165500	09-04-2003	RHEE; Chae-Seo et al.	
		033	US-20050261181	11-24-2005	WU; Dianqing et al.	
		034	US-20060127393	06-15-2006	LI; Ji et al.	
		035	US-20060263791	11-23-2006	MOON; Randall Todd et al.	
		036	US-20070196872	08-23-2007	BEX; Frederick et al.	
		037	US-20070207522	09-06-2007	LAURIE; Gordon et al.	
		038	US-20080038272	02-14-2008	BUEHRING; Hans-Joerg et al.	
		039	US-20080131435	06-05-2008	STAVENHAGEN; Jeffrey et al.	
		040	US-20080138344	06-12-2008	STAVENHAGEN; Jeffrey et al.	
		041	US-20080267955	10-30-2008	SCHLUESENER; Hermann et al.	
		042	US-20080286261	11-20-2008	MORGAN; Bruce A. et al.	
		043	US-20090028869	01-29-2009	DODEL; Richard et al.	
		044	US-20090092599	04-09-2009	LAZAR; Gregory Alan et al.	

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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		045	US-20090291088	11-26-2009	HARIHARAN; Kandasamy et al.	
		046	US-20090311243	12-17-2009	BROCKBANK; Sarah et al.	
		047	US-20100104574	04-29-2010	GURNEY; Austin L. et al.	
		048	US-20100129375	05-27-2010	JUNGE; Harald J. et al.	
		049	US-20100254979	10-07-2010	STAUNTON; Donald E. et al.	
		050	US-20100254980	10-07-2010	CONG; Feng et al.	
		051	US-20110105606	05-05-2011	RABBANI; Elazar et al.	
		052	US-20110177073	07-21-2011	VAN BERKEL; Patrick H. C. et al.	
		053	US-20110223140	09-15-2011	PARK; Sung Sup et al.	
		054	US-20110243963	10-06-2011	ABO; Arie et al.	
		055	US-20120237523	09-20-2012	MASCOLA; John R. et al.	
		056	US-20120322717	12-20-2012	LIU; Dakai et al.	
		057	US-20130058934	03-07-2013	CONG; Feng et al.	
		058	US-20130064823	03-14-2013	CONG; Feng et al.	
		059	US-20130095097	04-18-2013	BLANKENSHIP; John W. et al.	
		060	US-20130095104	04-18-2013	CUMMINGS; W. Jason et al.	
		061	US-20130183320	07-18-2013	WU; Yan et al.	
		062	US-20130230521	09-05-2013	NAKAMURA; Yusuke et al.	
		063	US-20130253172	09-26-2013	GURNEY; Austin L. et al.	
		064	US-20130274215	10-17-2013	THIES; R. Scott et al.	
		065	US-20130295105	11-07-2013	GURNEY; Austin L. et al.	

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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		066	US-20140044713	02-13-2014	DE LAU; Willibrordus Barend Maria et al.	
		067	US-20140050725	02-20-2014	JENKINS; David et al.	
		068	US-20140105917	04-17-2014	GURNEY; Austin L.	
		069	US-20140154252	06-05-2014	THOMPSON; Peter Armstrong et al.	
		070	US-20140200179	07-17-2014	GARCIA; Kenan Christopher et al.	
		071	US-20140242078	08-28-2014	DUPONT; Jakob et al.	
		072	US-20140363439	12-11-2014	BOURHIS; Eric et al.	
		073	US-20150010560	01-08-2015	XU; Cen et al.	
		074	US-20150119555	04-30-2015	JUNG; Gundram et al.	
		075	US-20150196663	07-16-2015	SHUSTA; Eric V. et al.	
		076	US-20150209407	07-30-2015	PIGNOLO; Robert J.	
		077	US-20150232554	08-20-2015	GURNEY; Austin L. et al.	
		078	US-20150266947	09-24-2015	SIERKS; Michael et al.	
		079	US-20150376252	12-31-2015	XU; Huaqiang Eric et al.	
		080	US-20160002312	01-07-2016	ILAN; Yaron	
		081	US-20160024196	01-28-2016	MAJETI; Jiangwen et al.	
		082	US-20160152947	06-02-2016	PIOSZAK; Augen A.	
		083	US-20160194394	07-07-2016	SIDHU; Sachdev et al.	
		084	US-20160264960	09-15-2016	ISHII; Shinya	
		085	US-20160312207	10-27-2016	KUO; Calvin J. et al.	
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Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		087	US-20170218077	08-03-2017	RAUM; Tobias et al.	
		088	US-20170218078	08-03-2017	RAUM; Tobias et al.	
		089	US-20170240631	08-24-2017	MONROE; Kate et al.	
		090	US-20170253670	09-07-2017	KLEIN; Christian et al.	
		091	US-20170306029	10-26-2017	GARCIA; Kenan Christopher et al.	
		092	US-20170349659	12-07-2017	GARCIA; Kenan Christopher et al.	
		093	US-20170355756	12-14-2017	JULIEN; Jean-Pierre et al.	
		094	US-20180066067	03-08-2018	CONG; Feng et al.	
		095	US-20190093072	03-28-2019	KOEHLER; Karl R. et al.	
		096	US-20190093079	03-28-2019	LOOSE; Christopher et al.	
		097	US-20200024338	01-23-2020	LUCA; Vincent Christopher et al.	
		098	US-20200048324	02-13-2020	ZHANG; Zhengjian et al.	
		099	US-20200199237	06-25-2020	GARCIA; Kenan Christopher et al.	
		100	US-20200199238	06-25-2020	GARCIA; Kenan Christopher et al.	
		101	US-20200308287	10-01-2020	LI; Yang et al.	
		102	US-20210079089	03-18-2021	LI; Yang et al.	
		103	US-20210087280	03-25-2021	LI; Yang et al.	
		104	US-20210292422	09-23-2021	LI; Yang	
		105	US-20210380678	12-09-2021	ZHANG; Zhengjian et al.	
		106	US-20210403578	12-30-2021	GARCIA; Kenan Christopher et al.	
		107	US-20220064337	03-03-2022	LI; Yang et al.	
		108	US-20220112278	04-14-2022	LI; Yang et al.	

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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		110	US-20220195053	06-23-2022	LI; Yang et al.	
		111	US-20220275095	09-01-2022	LI; Yang et al.	
		112	US-20230138045	05-04-2023	LI; Yang	
		113	US-20230183359	06-15-2023	GARCIA; Kenan Christopher et al.	

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Examiner Initials*		Cite No.[1]	Foreign Patent Document Country Code[3]Number[4]Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
		114	CN-101951954-A	01-19-2011	NOVARTIS AG.		☒
		115	CN-103002911-A	03-27-2013	ONCOMED PHARM INC.		☒
		116	CN-103998462-A	08-20-2014	FATE THERAPEUTICS INC.		☒
		117	EP-1716181-B1	12-16-2009	GENENTECH INC.		☒
		118	EP-2910550-A2	08-26-2015	ENZO THERAPEUTICS INC.		☐
		119	EP-3191526-A4	03-28-2018	UNIV LELAND STANFORD JUNIOR., et al.		☐

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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		120	JP-2011503025-A	01-27-2011	NOVARTIS AG.		☒
		121	JP-2012503990-A	02-16-2012	ONCOMED PHARMACEUTICALS INC.		☒
		122	JP-2012506568-A	03-15-2012	CAMBRIDGE DISPLAY TECH LTD.		☒
		123	JP-2012516685-A	07-26-2012	KONINK NL AKADEMIE VAN WETENSC.		☒
		124	JP-2013527761-A	07-04-2013	NOVARTIS AG.		☒
		125	JP-2017530099-A	10-12-2017	THE BOARD OF TRUSTEES OF THE LELAND STANFORD JR. UNIVERSITY.		☒
		126	WO-02092635-A2	11-21-2002	UNIV CALIFORNIA.		☐
		127	WO-2004063351-A2	07-29-2004	MACROGENICS INC., et al.		☐
		128	WO-2005032574-A1	04-14-2005	UNIV LELAND STANFORD JUNIOR., et al.		☐
		129	WO-2006040163-A1	04-20-2006	LINIKUM EBERHARD KARLS UNI TUE., et al.		☒
		130	WO-2006079372-A1	08-03-2006	ABLYNX NV., et al.		☐

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Application Number	18/361,782
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First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
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		131	WO-2006088494-A2	08-24-2006	MACROGENICS INC., et al.		☐
		132	WO-2006105338-A2	10-05-2006	XENCOR INC., et al.		☐
		133	WO-2007012449-A1	02-01-2007	PHASYS GMBH M., et al.		☒
		134	WO-2007024249-A2	03-01-2007	MACROGENICS INC., et al.		☐
		135	WO-2007146968-A2	12-21-2007	TRUBION PHARMACEUTICALS., et al.		☐
		136	WO-2007148417-A1	12-27-2007	ONCOTHERAPY SCIENCE INC., et al.		☐
		137	WO-2008068048-A2	06-12-2008	IST SUPERIORE SANITA., et al.		☐
		138	WO-2008084402-A2	07-17-2008	UNIV MARBURG PHILIPPS., et al.		☐
		139	WO-2008134632-A1	11-06-2008	HARVARD COLLEGE., et al.		☐
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		141	WO-2009064944-A2	05-22-2009	NUVELO INC., et al.		☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

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		142	WO-2009080251-A1	07-02-2009	HOFFMANN LA ROCHE., et al.		☐
		143	WO-2010016766-A2	02-11-2010	KONINK NL AKADEMIE VAN WETENSC., et al.		☐
		144	WO-2010021697-A2	02-25-2010	PFIZER., et al.		☐
		145	WO-2010037041-A2	04-01-2010	ONCOMED PHARM INC., et al.		☐
		146	WO-2010054010-A1	05-14-2010	FABRUS LLC., et al.		☐
		147	WO-2010090513-A2	08-12-2010	KONINK NL AKADEMIE VAN WETENSC., et al.		☐
		148	WO-2011088226-A2	07-21-2011	CARIS LIFE SCIENCES LUXEMBOURG HOLDINGS., et al.		☐
		149	WO-2011090762-A1	07-28-2011	EMERGENT PRODUCT DEV SEATTLE., et al.		☐
		150	WO-2011119661-A1	09-29-2011	GENENTECH INC., et al.		☐
		151	WO-2011123785-A2	10-06-2011	ONCOMED PHARM INC., et al.		☐
		152	WO-2011138392-A1	11-10-2011	NOVARTIS AG., et al.		☐

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Art Unit	Not Yet Assigned
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		153	WO-2012014076-A2	02-02-2012	KONINKLIJKE NEDERLANDSE AKADEMIE VAN WETENSCHAPPEN., et al.		☐
		154	WO-2012058768-A1	05-10-2012	ZYMEWORKS INC., et al.		☐
		155	WO-2012103360-A2	08-02-2012	UNIV LELAND STANFORD JUNIOR., et al.		☐
		156	WO-2012140274-A2	10-18-2012	KONINKLIJKE NEDERLANDSE AKADEMIE VAN WETENSCHAPPEN., et al.		☐
		157	WO-2012140274-A9	03-07-2013	KONINKLIJKE NEDERLANDSE AKADEMIE VAN WETENSCHAPPEN., et al.		☐
		158	WO-2013092001-A1	06-27-2013	SYNIMMUNE GMBH.		☐
		159	WO-2013109819-A1	07-25-2013	GENENTECH INC., et al.		☐
		160	WO-2014029752-A1	02-27-2014	GLAXO GROUP LTD.		☐
		161	WO-2014124326-A1	08-14-2014	STEM CENTRX INC.		☐

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		162	WO-2014143022-A1	09-18-2014	TRESE MICHAEL T., et al.		☐
		163	WO-2014159580-A1	10-02-2014	ONCOMED PHARM INC.		☐
		164	WO-2015036582-A2	03-19-2015	KIPRIJANOV SERGEJ MICHAILOVIC.		☐
		165	WO-2015063187-A1	05-07-2015	KIPRIJANOV SERGEJ MICHAILOVIC.		☐
		166	WO-2015109212-A1	07-23-2015	PFIZER., et al.		☐
		167	WO-2016023019-A2	02-11-2016	ALECTOR LLC.		☐
		168	WO-2016040895-A1	03-17-2016	THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY, et al.		☐
		169	WO-2016168607-A1	10-20-2016	UNIV TENNESSEE RES FOUND.		☐
		170	WO-2016205551-A2	12-22-2016	UNIV CALIFORNIA.		☐
		171	WO-2016205566-A1	12-22-2016	UNIV CALIFORNIA., et al.		☐

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		172	WO-2017127933-A1	08-03-2017	GOVERNING COUNCIL UNIV TORONTO.		☐
		173	WO-2017136820-A2	08-10-2017	EPIMAB BIOTHERAPEUTICS INC., et al.		☐
		174	WO-2017152102-A2	09-08-2017	ALECTOR LLC.		☐
		175	WO-2018132572-A1	07-19-2018	THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY.		☐
		176	WO-2018140821-A1	08-02-2018	SURROZEN INC.		☐
		177	WO-2018220080-A1	12-06-2018	BOEHRINGER INGELHEIM INT.		☐
		178	WO-2019126398-A1	06-27-2019	SURROZEN INC.		☐
		179	WO-2019126399-A1	06-27-2019	SURROZEN INC.		☐
		180	WO-2019126401-A1	06-27-2019	SURROZEN INC.		☐
		181	WO-2019159084-A1	08-22-2019	ANGERS STEPHANE., et al.		☐
		182	WO-2020010308-A1	01-09-2020	SURROZEN INC.		☐

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		183	WO-2020014271-A1	01-16-2020	SURROZEN INC.		☐
		184	WO-2020132356-A1	06-25-2020	SURROZEN INC.		☐
		185	WO-2020167848-A1	08-20-2020	SURROZEN INC.		☐
		186	WO-2020185960-A1	09-17-2020	SURROZEN INC., et al.		☐
		187	WO-2020206005-A1	10-08-2020	SURROZEN INC.		☐
		188	WO-2021003054-A1	01-07-2021	RAMPART COMMUNICATIONS INC.		☐
		189	WO-2021003416-A1	01-07-2021	SURROZEN INC.		☐
		190	WO-2021173726-A1	09-02-2021	SURROZEN INC.		☐
		191	WO-2022104280-A1	05-19-2022	SURROZEN OPERATING INC.		☐
		192	WO-2022192445-A1	09-15-2022	SURROZEN OPERATING INC.		☐
		193	WO-2023044348-A1	03-23-2023	SURROZEN OPERATING INC.		☐

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Examiner Name	Not Yet Assigned
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		194	WO-2023115048-A1	06-22-2023	SURROZEN OPERATING INC.		☐
		195	WO-2023130055-A2	07-06-2023	SURROZEN OPERATING INC.		☐
		196	WO-9627011-A1	09-06-1996	GENENTECH INC.		☐
		197	WO-9850431-A2	11-12-1998	GENENTECH INC.		☐

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		218	BASIL et al., (2022) "Human distal airways contain a multipotent secretory cell that can regenerate alveoli". Nature, 604(7904): 120-126.	☐
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		220	BERGSTRÖM, J.H. et al. (2014) "AGR2, an endoplasmic reticulum protein, is secreted into the gastrointestinal mucus" PLoS One, 9(8):e104186, 1-8.	☐
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Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

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		228	BRAMHALL et al. (2014). "Lgr5-Positive Supporting Cells Generate New Hair Cells in the Postnatal Cochlea." Stem Cell Reports. 2(3): 311-322.	☐
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		237	CHEN et al. (2009). "Aminoglycoside-induced histone deacetylation and hair cell death in the mouse cochlea," J. Neurochem., 108(5): 1226-1236.	☐
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		246	CHILOSI et al. (2003) "Aberrant Wnt/β-catenin pathway activation in idiopathic pulmonary fibrosis". The American Journal of Pathology, 162(5): 1495-1502.	☐
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Sheet 22 of 41

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		276	GenBank Accession No. NM_014421.3 "Homo sapiens dickkopf WNT signaling pathway inhibitor 2 (DKK2), mRNA" February 13, 2021, 4 pages.	☐
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Sheet 35 of 41

Complete if Known	
Application Number	18/361,782
Filing Date	07-28-2023
First Named Inventor	LI; Yang
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	SRZN-006/04US 328202-2203

NON-PATENT LITERATURE DOCUMENTS

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		399	SCHUIJERS et al. (2012) Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. EMBO J, 31:2685-2696.	☐
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		401	SCHWITALLA, S. et al. (2013 Jan) Intestinal Tumorigenesis Initiated by Dedifferentiation and Acquisition of Stem-Cell-like Properties. Cell, 152(1-2):25-38; https://doi.org/10.1016/j.cell.2012.12.012.	☐
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		404	SEMENOV, M.V. et al. (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr Biol, 11:951-961.	☐
		405	SHI, F. et al. (2012 Jul) Wnt-Responsive Lgr5-Expresesing Stem Cells Are Hair Cell Progenitors in the Cochlea. J Neurosci, 32(28):9639-9648.	☐
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		432	WIRTZ, S. et al. (2017). Chemically induced mouse models of acute and chronic intestinal inflammation. Nature Protocols, 12(7), 1295-1309. https://doi.org/10.1038/nprot.2017.044.	☐
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		451	ZOUKHRI "Mechanisms Involved in Injury and Repair of the Murine Lacrimal Gland: Role of Programmed Cell Death and Mesenchymal Stem Cells", Ocul Surf., (2010); 8(2):60-69.	☐

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Bibliographic data

Title:	Wnt compositions and therapeutic uses of such compositions
Pub/Pat no:	CN103998462A
Pub/Issue Date:	2014-08-20
Inventor(s):	LEE TOM TONG| FITCH MICHAEL J| LAI KEVIN| BENNETT MONICA| FLYNN PETER|LEE TOM TONG |FITCH MICHAEL J |LAI KEVIN |BENNETT MONICA |FLYNN PETER
Applicant(s):	FATE THERAPEUTICS INC
Classification:	A61K38/17AI; C07K14/47AI; C07K19/00AI; C12N15/12AI; C12N15/63AI
Application number:	CN20128049247 2012-09-14
Priority number:	US201161535913P 20110916; WO2012US55336 20120914;

Abstract of CN103998462A

The invention provides novel Wnt polypeptides that have improved production characteristics, solubility, systemic delivery, and tissue uptake, and polynucleotides encoding the Wnt polypeptides of the invention. The Wnt polypeptides of the invention can be used therapeutically, such as, for example, in methods of preventing or treating muscle loss and/or promoting muscle hypertrophy and growth.

(19)中华人民共和国国家知识产权局

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代理人 王达佐 洪欣
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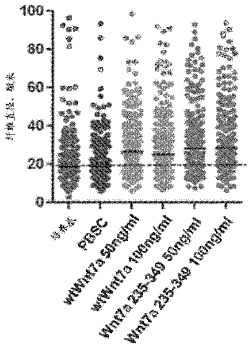
权利要求书5页 说明书44页
序列表47页 附图32页

(54)发明名称

WNT组合物及此类组合物的治疗应用

(57)摘要

本发明提供新的Wnt多肽和编码本发明的Wnt多肽的多核苷酸，所述Wnt多肽具有改良的生产特性、溶解度、全身性递送和组织吸收。本发明的Wnt多肽可在治疗上用于例如，预防或治疗肌肉减少和/或促进肌肉肥大和生长的方法中。



CN 103998462 A

1. 包含 N 端缺失的分离的 Wnt 多肽，其中所述 N 端缺失去除了一个或多个脂化位点。

2. 根据权利要求 1 所述的分离的 Wnt 多肽，其中所述 N 端缺失包含 300 个 N 端氨基酸的缺失。

3. 根据权利要求 1 所述的分离的 Wnt 多肽，其中所述 N 端缺失包含 250 个 N 端氨基酸的缺失。

4. 根据权利要求 1 所述的分离的 Wnt 多肽，其中所述 N 端缺失包含 200 个 N 端氨基酸的缺失。

5. 根据权利要求 1 所述的分离的 Wnt 多肽，其中所述 N 端缺失包含 150 个 N 端氨基酸的缺失。

6. 根据权利要求 1-5 中任一项所述的分离的 Wnt 多肽，其中所述 N 端缺失去除了一个脂化位点。

7. 根据权利要求 1-5 中任一项所述的分离的 Wnt 多肽，其中所述 N 端缺失去除了至少两个脂化位点。

8. 根据权利要求 1-5 中任一项所述的分离的 Wnt 多肽，其中所述 N 端缺失去除了所有脂化位点。

9. 根据权利要求 1-8 中任一项所述的分离的 Wnt 多肽，其中所述分离的 Wnt 多肽还包含一个或多个 C 端氨基酸的 C 端缺失。

10. 根据权利要求 1-8 中任一项所述的分离的 Wnt 多肽，其中所述分离的 Wnt 多肽还包含至少 10 个 C 端氨基酸的 C 端缺失。

11. 根据权利要求 1-8 中任一项所述的分离的 Wnt 多肽，其中所述分离的 Wnt 多肽还包含至少 20 个 C 端氨基酸的 C 端缺失。

12. 根据权利要求 1-8 中任一项所述的分离的 Wnt 多肽，其中所述分离的 Wnt 多肽还包含至少 50 个 C 端氨基酸的 C 端缺失。

13. 根据权利要求 1-12 中任一项所述的分离的 Wnt 多肽，其中所述多肽包含具有生物活性的 Wnt 多肽。

14. 根据权利要求 1-13 中任一项所述的分离的 Wnt 多肽，其中所述多肽保留了非经典的 Wnt 信号转导活性。

15. 根据权利要求 1-14 中任一项所述的分离的 Wnt 多肽，其中所述多肽相比天然存在的 Wnt 多肽具有改良的产品收率。

16. 根据权利要求 1-14 中任一项所述的分离的 Wnt 多肽，其中所述多肽相比天然存在的 Wnt 多肽具有改良的分泌特性。

17. 根据权利要求 1-14 中任一项所述的分离的 Wnt 多肽，其中所述多肽相比天然存在的 Wnt 多肽具有改良的稳定性或半衰期。

18. Wnt 融合多肽，其包含权利要求 1-17 中任一项所述分离的 Wnt 多肽。

19. 根据权利要求 18 所述的 Wnt 融合多肽，其包含 Fc 结构域。

20. 根据权利要求 19 所述的 Wnt 融合多肽，其中所述多肽不具有抗体依赖性细胞介导的细胞毒性 (ADCC) 或补体依赖性细胞毒性 (CDC) 活性。

21. 根据权利要求 19 所述的 Wnt 融合多肽，其中所述多肽相比天然存在的 Wnt 多肽具有改良的产品收率。

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22. 根据权利要求 19 所述的 Wnt 融合多肽，其中所述多肽相比天然存在的 Wnt 多肽具有改良的分泌特性。

23. 根据权利要求 19 所述的 Wnt 融合多肽，其中所述多肽相比天然存在的 Wnt 多肽具有改良的稳定性或半衰期。

24. 根据权利要求 18-23 中任一项所述的 Wnt 融合多肽，其包含天然信号肽、异源信号肽，或天然和异源信号肽的杂合体。

25. 根据权利要求 24 所述的 Wnt 融合多肽，其中所述异源信号肽选自：CD33 信号肽、免疫球蛋白信号肽、生长激素信号肽、红细胞生成素信号肽、白蛋白信号肽、分泌型碱性磷酸酶信号肽和病毒信号肽。

26. 根据权利要求 25 所述的 Wnt 融合多肽，其中所述异源信号肽为 CD33 信号肽、IgG κ 信号肽或 IgG μ 信号肽。

27. 根据权利要求 18-26 中任一项所述的 Wnt 融合多肽，其包含异源蛋白酶切割位点。

28. 根据权利要求 27 所述的 Wnt 融合多肽，其中所述异源蛋白酶切割位点选自：烟草蚀纹病毒 (TEV) 蛋白酶切割位点、肝素切割位点、凝血酶切割位点、肠激酶切割位点和 Xa 因子切割位点。

29. 根据权利要求 18-28 中任一项所述的 Wnt 融合多肽，其包含选自以下的表位标签：HIS6 表位、MYC 表位、FLAG 表位、V5 表位、VSV-G 表位和 HA 表位。

30. 组合物，其包含权利要求 1-17 中任一项所述的 Wnt 多肽或权利要求 18-29 中任一项所述的 Wnt 融合多肽。

31. 根据权利要求 30 所述的组合物，其包含药学上可接受的盐、载体或赋形剂。

32. 根据权利要求 31 所述的组合物，其中赋形剂增加了所述组合物的 Wnt 多肽或 Wnt 融合多肽的半衰期。

33. 根据权利要求 31 所述的组合物，其中赋形剂增加了所述组合物的 Wnt 多肽或 Wnt 融合多肽的稳定性。

34. 分离的 Wnt7a 多肽，其包含：

(a) 与 SEQ ID NO:2 所示氨基酸序列具有至少 80% 同一性的氨基酸序列，并且还包含 SEQ ID NO:2 所示氨基酸序列的至少 220 个氨基酸的 N 端缺失；

(b) 与 SEQ ID NO:3 所示氨基酸序列具有至少 80% 同一性的氨基酸序列；

(c) 与 SEQ ID NO:4 所示氨基酸序列具有至少 85% 同一性的氨基酸序列；

(d) 与 SEQ ID NO:5 所示氨基酸序列具有至少 80% 同一性的氨基酸序列；或

(e) 包含与 SEQ ID NO:3-5 中任一序列所示氨基酸序列相同的至少 70 个连续氨基酸的氨基酸序列。

35. 根据权利要求 34 所述的分离的 Wnt7a 多肽，其中所述分离的 Wnt7a 多肽还包含一个或多个 C 端氨基酸的 C 端缺失。

36. 根据权利要求 34 所述的分离的 Wnt7a 多肽，其中所述分离的 Wnt7a 多肽还包含至少 10 个 C 端氨基酸的 C 端缺失。

37. 根据权利要求 34 所述的分离的 Wnt7a 多肽，其中所述分离的 Wnt7a 多肽还包含至少 20 个 C 端氨基酸的 C 端缺失。

38. 根据权利要求 34-37 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽包含具有生

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物活性的 Wnt7a 多肽。

39. 根据权利要求 34-38 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽保留了非经典的 Wnt7a 信号转导活性。

40. 根据权利要求 34-39 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽相比天然存在的 Wnt7a 多肽具有改良的产品收率。

41. 根据权利要求 34-39 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽相比天然存在的 Wnt7a 多肽具有改良的分泌特性。

42. 根据权利要求 34-39 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽相比天然存在的 Wnt7a 多肽具有改良的稳定性或半衰期。

43. 根据权利要求 34 所述的分离的 Wnt7a 多肽，其中所述多肽包含 SEQ ID NO:3-5 中任一序列所示的氨基酸序列。

44. 根据权利要求 34 所述的分离的 Wnt7a 多肽，其中所述多肽包含 SEQ ID NO:3 所示氨基酸序列的至少 60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128 或 129 个连续氨基酸。

45. 根据权利要求 34-44 中任一项所述的分离的 Wnt7a 多肽，其中所述分离的 Wnt7a 多肽相比具有 SEQ ID NO:2 和 18-23 中任一序列所示氨基酸序列的 Wnt 多肽，具有增加的水溶液溶解度。

46. 根据权利要求 34-45 中任一项所述的分离的 Wnt7a 多肽，其中所述分离的 Wnt7a 多肽结合细胞表面上的卷曲受体。

47. 根据权利要求 34-46 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽保留了非经典的 Wnt 信号转导活性。

48. 根据权利要求 34-47 中任一项所述的分离的 Wnt7a 多肽，其中所述多肽未被脂化并且保留了非经典的 Wnt 信号转导活性。

49. 根据权利要求 46 所述的分离的 Wnt7a 多肽，其中所述细胞为骨骼肌卫星干细胞。

50. 根据权利要求 46 所述的分离的 Wnt7a 多肽，其中相比缺少所述分离的 Wnt7a 多肽下的卫星干细胞扩增，所述分离的 Wnt7a 多肽与卷曲受体的结合增加了卫星干细胞扩增。

51. Wnt7a 融合多肽，其包含权利要求 34-50 中任一项所述的分离的 Wnt7a 多肽。

52. 根据权利要求 51 所述的 Wnt7a 融合多肽，其包含 Fc 结构域。

53. 根据权利要求 52 所述的 Wnt7a 融合多肽，其中所述肽不具有 ADCC 或 CDC 活性。

54. 根据权利要求 51-53 中任一项所述的 Wnt7a 融合多肽，其中所述多肽相比天然存在的 Wnt7a 多肽具有改良的产品收率。

55. 根据权利要求 51-53 中任一项所述的 Wnt7a 融合多肽，其中所述多肽相比天然存在的 Wnt7a 多肽具有改良的分泌特性。

56. 根据权利要求 51-53 中任一项所述的 Wnt7a 融合多肽，其中所述多肽相比天然存在的 Wnt7a 多肽具有改良的稳定性或半衰期。

57. 根据权利要求 51-56 中任一项所述的 Wnt7a 融合多肽，其包含天然信号肽、异源信号肽，或天然和异源信号肽的杂合体。

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58. 根据权利要求 57 所述的 Wnt7a 融合多肽，其中所述异源信号肽选自：CD33 信号肽、免疫球蛋白信号肽、生长激素信号肽、红细胞生成素信号肽、白蛋白信号肽、分泌型碱性磷酸酶信号肽和病毒信号肽。

59. 根据权利要求 57 所述的 Wnt7a 融合多肽，其中所述异源信号肽为 CD33 信号肽、IgGκ 信号肽或 IgGμ 信号肽。

60. 根据权利要求 51-59 中任一项所述的 Wnt7a 融合多肽，其包含异源蛋白酶切割位点。

61. 根据权利要求 60 所述的 Wnt7a 融合多肽，其中所述异源蛋白酶切割位点选自：烟草蚀纹病毒（TEV）蛋白酶切割位点、肝素切割位点、凝血酶切割位点、肠激酶切割位点和 Xa 因子切割位点。

62. 根据权利要求 51-61 中任一项所述的 Wnt7a 融合多肽，其包含选自以下的表位标签：HIS6 表位、MYC 表位、FLAG 表位、V5 表位、VSV-G 表位和 HA 表位。

63. 多核苷酸，其编码权利要求 34-50 中任一项所述的 Wnt7a 多肽或权利要求 51-62 中任一项所述的 Wnt7a 融合多肽。

64. 载体，其包含权利要求 63 所述的多核苷酸。

65. 宿主细胞，其包含权利要求 64 所述的载体。

66. 根据权利要 65 所述的宿主细胞，其中所述宿主细胞为哺乳动物细胞或细菌细胞。

67. 由权利要 66 所述的宿主细胞产生的 Wnt7a 多肽或 Wnt7a 融合多肽。

68. 组合物，其包含：权利要求 34-50 中任一项所述的 Wnt7a 多肽或权利要求 51-62 中任一项所述的 Wnt7a 融合多肽；编码权利要求 34-50 中任一项所述的 Wnt7a 多肽或权利要求 51-62 中任一项所述的 Wnt7a 融合多肽的多核苷酸；或者含有编码权利要求 34-50 中任一项所述的 Wnt7a 多肽或权利要求 51-62 中任一项所述的 Wnt7a 融合多肽的多核苷酸的载体。

69. 根据权利要求 68 所述的组合物，其包含药学上可接受的盐、载体或赋形剂。

70. 根据权利要求 68 所述的组合物，其中赋形剂增加了所述组合物的 Wnt7a 多肽或 Wnt7a 融合多肽的半衰期。

71. 根据权利要求 68 所述的组合物，其中赋形剂增加了所述组合物的 Wnt7a 多肽或 Wnt7a 融合多肽的稳定性。

72. 根据权利要求 68 所述的组合物，其中所述组合物可溶于水溶液。

73. 根据权利要求 68 所述的组合物，其中所述组合物经配制用于注射。

74. 根据权利要求 68 所述的组合物，其中所述组合物经配制用于以下的一种或多种：静脉内注射、心脏内注射、皮下注射、腹腔内注射或直接注射至肌肉。

75. 根据权利要求 68 所述的组合物，其中所述组合物促进组织形成、再生、维持或修复。

76. 根据权利要求 75 所述的组合物，其中所述组织为肌肉。

77. 根据权利要求 76 所述的组合物，其中所述肌肉为骨骼肌、心肌或平滑肌。

78. 根据权利要求 68 所述的组合物，其中所述组合物促进干细胞扩增。

79. 根据权利要求 78 所述的组合物，其中所述干细胞为成体干细胞。

80. 根据权利要求 79 所述的组合物，其中所述成体干细胞为卫星干细胞。

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81. 根据权利要求 76 所述的组合物，其中所述组合物促进肌肉肥大或预防萎缩。

82. 治疗或预防肌肉减少的方法，其包括将权利要求 68 所述的组合物给予至对象。

83. 根据权利要求 82 所述的方法，其中所述组合物可溶于水溶液。

84. 根据权利要求 82 所述的方法，其中所述组合物经配制用于注射。

85. 根据权利要求 82 所述的方法，其中所述组合物经配制用于以下的一种或多种：静脉内注射、心脏内注射、皮下注射、腹腔内注射或直接注射至肌肉。

86. 根据权利要求 82 所述的方法，其中所述对象患有影响肌肉的疾病或病症，或者有患影响肌肉的疾病或病症的风险。

87. 根据权利要求 86 所述的方法，其中所述疾病为退行性疾病。

88. 根据权利要求 87 所述的方法，其中所述退行性疾病为肌肉萎缩症。

89. 根据权利要求 88 所述的方法，其中所述肌肉萎缩症选自：杜氏肌肉萎缩症 (DMD)、贝克氏肌肉萎缩症 (BMD)、埃 - 德二氏肌肉萎缩症、兰 - 迪二氏肌肉萎缩症、面肩胛臂肌肉萎缩症 (FSH)、肢 - 带型肌肉萎缩症、冯 • 格雷非 - 福克斯肌肉萎缩症、眼咽型肌肉萎缩症 (OPMD)、强直性肌肉萎缩症（斯坦纳特病）和先天性肌肉萎缩症。

90. 根据权利要求 86 所述的方法，其中所述影响肌肉的疾病或病症为消耗性疾病、肌肉衰减、肌肉萎缩、ICU 引起的虚弱、长期废用性肌萎缩、手术引起的虚弱或肌肉退行性疾病。

91. 根据权利要求 86 所述的方法，其中所述病症是与肌肉废用、长期固定不动、手术引起的虚弱或损伤有关的肌肉萎缩症。

92. 根据权利要求 82 所述的方法，其中给予所述组合物预防肌肉萎缩。

93. 根据权利要求 82 所述的方法，其中给予所述组合物促进肌肉肥大。

94. 根据权利要求 92 或 93 所述的方法，其中所述肌肉为骨骼肌或心肌。

95. 根据权利要求 92 或 93 所述的方法，其中给予所述组合物促进卫星干细胞扩增。

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WNT 组合物及此类组合物的治疗应用

[0001] 相关申请的交叉引用

[0002] 本申请基于 35U.S.C. §119(e) 要求于 2011 年 9 月 16 日递交的美国临时申请第 61/535,913 号的权益，其通过引用整体并入本文。

[0003] 关于序列表的申明

[0004] 与本申请相关的序列表提供了文本格式以代替纸件副本，其在此通过引用并入本说明书中。含有该序列表的文本文件的名称为 FATE_109_01WO_ST25.txt。该文本文件大小为 76KB，创建于 2012 年 9 月 10 日，并通过 EFS-Web 电子递交。

[0005] 发明背景

技术领域

[0006] 本发明总体涉及 Wnt 组合物和使用其的治疗方法。本发明的 Wnt 多肽及其组合物可在治疗上用于，例如通过促进干细胞扩增和肌肉肥大来促进肌肉再生。

[0007] 相关技术描述

[0008] Wnt 家族基因编码超过 20 种富含半胱氨酸的分泌型 Wnt 糖蛋白，其通过结合至靶细胞上的卷曲 (Fzd) 受体而发挥作用。卷曲受体为 G 蛋白偶联受体蛋白家族。所述 Wnt 家族的不同成员结合至所述 Fzd 家族的某些成员能够通过几种不同通路中的一种来启动信号转导。在“经典通路”中，信号转导分子蓬乱蛋白 (Disheveled) 导致糖原合成酶激酶 -3(GSK3β)——细胞质丝氨酸 - 苏氨酸激酶的失活。GSK-3β 靶标——β - 连环蛋白因而得以稳定并转移至细胞核，在那里其激活特定启动子的 TCF(T- 细胞 - 因子) 依赖性转录 (Wodarz,1998,Dierick,1999)。“非经典的”Wnt 通路激活并未得到充分定义，但包括 Wnt 和 Fzd 之间的一部分的相互作用，其可激活 Ca2+ 通路信号转导和可能的 PI3K 信号转导、Rho 通路信号转导，以及平面细胞极性 (PCP) 通路信号转导。

[0009] Wnt 为分泌型糖蛋白，其作为旁分泌或自分泌信号活跃于几种原始细胞类型中。尽管 Wnt 蛋白分泌自细胞，但它们被发现为疏水性的，且被认为是通过在保守半胱氨酸残基和保守丝氨酸残基上添加脂质基团而进行了翻译后修饰。人们普遍认为这些脂质修饰对于 Wnt 蛋白的生物活性和分泌非常重要。然而，在配制过程中当未使用清洁剂时，脂化的 Wnt 的脂化和低溶解度与低的生产收率有关，并因此对 Wnt 的临床规模生产提供了独特的挑战。因此，虽然 Wnt 具有在各种临床情况下用作治疗剂的巨大潜力，由于 Wnt 的不溶性和相应的作为纯化的生物活性治疗剂的生产不足，Wnt 的治疗潜力还有待于充分实现。

[0010] 因此，本领域需要保留 Wnt 生物活性的可溶性 Wnt 多肽、在临床规模上生产所述 Wnt 的方法，以及应用所述 Wnt 来促进组织形成、再生、维持和修复的方法。

[0011] 发明概述

[0012] 本发明总体上提供新的 Wnt 组合物和用于通过促进干细胞扩增和肌肉肥大来促进肌肉再生的治疗方法。

[0013] 在一个实施方案中，本发明部分地包括包含 N 端缺失的分离的 Wnt 多肽，其中所述 N 端缺失去除了一个或多个脂化位点。

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[0014] 在一个具体实施方案中，所述 N 端缺失包含 300 个 N 端氨基酸的缺失。

[0015] 在另一个具体实施方案中，所述 N 端缺失包含 250 个 N 端氨基酸的缺失。

[0016] 在又一个具体实施方案中，所述 N 端缺失包含 200 个 N 端氨基酸的缺失。

[0017] 在又一个具体实施方案中，所述 N 端缺失包含 150 个 N 端氨基酸的缺失。

[0018] 在一个实施方案中，所述 N 端缺失去除了一个脂化位点。

[0019] 在另一个实施方案中，所述 N 端缺失去除了至少两个脂化位点。

[0020] 在又一个实施方案中，所述 N 端缺失去除了所有脂化位点。

[0021] 在一个相关的实施方案中，所述分离的 Wnt 多肽还包含一个或多个 C 端氨基酸的 C 端缺失。

[0022] 在另一个相关的实施方案中，所述分离的 Wnt 多肽还包含至少 10 个 C 端氨基酸的 C 端缺失。

[0023] 在又一个相关的实施方案中，所述分离的 Wnt 多肽还包含至少 20 个 C 端氨基酸的 C 端缺失。

[0024] 在又一个相关的实施方案中，所述分离的 Wnt 多肽还包含至少 50 个 C 端氨基酸的 C 端缺失。

[0025] 在一个具体实施方案中，所述分离的 Wnt 多肽包含具有生物活性的 Wnt 多肽。

[0026] 在某个具体的实施方案中，所述分离的 Wnt 多肽保留了非经典的 Wnt 信号转导活性。

[0027] 在相关的具体实施方案中，所述分离的 Wnt 多肽相比天然存在的 Wnt 多肽具有改良的产品收率。

[0028] 在又一个具体实施方案中，所述分离的 Wnt 多肽相比天然存在的 Wnt 多肽具有改良的分泌特性。

[0029] 在又一个具体实施方案中，所述 Wnt 多肽相比天然存在的 Wnt 多肽具有改良的稳定性或半衰期。

[0030] 在各种实施方案中，本发明部分地提供了 Wnt 融合多肽，其包含根据本文所述实施方案中任一个方案的分离的 Wnt 多肽。

[0031] 在一个实施方案中，所述 Wnt 融合多肽包含 Fc 结构域。

[0032] 在另一个实施方案中，所述 Wnt 融合多肽不具有抗体依赖性细胞介导的细胞毒性 (ADCC) 或补体依赖性细胞毒性 (CDC) 活性。

[0033] 在一个具体实施方案中，相比天然存在的 Wnt 多肽，所述 Wnt 融合多肽具有改良的产品收率。

[0034] 在某个实施方案中，相比天然存在的 Wnt 多肽，所述 Wnt 融合多肽具有改良的分泌特性。

[0035] 在某个具体的实施方案中，相比天然存在的 Wnt 多肽，所述 Wnt 融合多肽具有改良的稳定性或半衰期。

[0036] 在某个另外的实施方案中，所述 Wnt 融合多肽包含天然信号肽、异源信号肽，或天然和异源信号肽的杂合体。

[0037] 在另外的某个实施方案中，所述 Wnt 融合多肽包含选自以下的异源信号肽 :CD33 信号肽、免疫球蛋白信号肽、生长激素信号肽、红细胞生成素信号肽、白蛋白信号肽、分泌型

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碱性磷酸酶信号肽和病毒信号肽。

[0038] 在另一个实施方案中，所述异源信号肽为 CD33 信号肽、IgGκ 信号肽或 IgGμ 信号肽。

[0039] 在另一个相关的实施方案中，所述 Wnt 融合多肽包含异源蛋白酶切割位点。

[0040] 在具体的相关实施方案中，所述异源蛋白酶切割位点选自：烟草蚀纹病毒 (TEV) 蛋白酶切割位点、肝素切割位点、凝血酶切割位点、肠激酶切割位点和 Xa 因子切割位点。

[0041] 在一个实施方案中，所述 Wnt 融合多肽包含选自以下的表位标签：HIS6 表位、MYC 表位、FLAG 表位、V5 表位、VSV-G 表位和 HA 表位。

[0042] 在各种实施方案中，本发明部分地包括这样的组合物，其包含根据本文公开的实施方案中任一个方案的 Wnt 多肽，或根据本文公开的实施方案中任一个方案的 Wnt 融合多肽。

[0043] 在具体的实施方案中，所述组合物包含药学上可接受的盐、载体或赋形剂。

[0044] 在某个实施方案中，所述赋形剂增加了所述组合物的 Wnt 多肽或 Wnt 融合多肽的半衰期。

[0045] 在又一个实施方案中，所述赋形剂增加了所述组合物的 Wnt 多肽或 Wnt 融合多肽的稳定性。

[0046] 在一个实施方案中，本发明部分地包括分离的 Wnt7a 多肽，其包含：与 SEQ ID NO:2 所示氨基酸序列有至少 80% 的同一性的氨基酸序列，并且还包含 SEQ ID NO:2 所示氨基酸序列的至少 220 个氨基酸的 N- 端缺失；与 SEQ ID NO:3 所示氨基酸序列有至少 80% 的同一性的氨基酸序列；与 SEQ ID NO:4 所示氨基酸序列有至少 85% 的同一性的氨基酸序列；与 SEQ ID NO:5 所示氨基酸序列有至少 80% 的同一性的氨基酸序列；或包含与 SEQ ID NO:3-5 中任一序列所示氨基酸序列相同的至少 70 个连续氨基酸的氨基酸序列。

[0047] 在一个具体实施方案中，分离的 Wnt7a 多肽包含：与 SEQ ID NO:2 所示氨基酸序列有至少 95% 的同一性的氨基酸序列，并且还包含 SEQ ID NO:2 所示氨基酸序列的至少 220 个氨基酸的 N 端缺失；与 SEQ ID NO:3 所示氨基酸序列有至少 95% 的同一性的氨基酸序列；与 SEQ ID NO:4 所示氨基酸序列有至少 95% 的同一性的氨基酸序列；与 SEQ ID NO:5 所示氨基酸序列有至少 95% 的同一性的氨基酸序列；或包含与 SEQ ID NO:3-5 中任一序列所示氨基酸序列相同的至少 70 个连续氨基酸的氨基酸序列。

[0048] 在某个实施方案中，分离的 Wnt7a 多肽包含：可与 SEQ ID NO:3 的序列进行最优比对而产生至少 220 的相似性得分的氨基酸序列，比对使用 BLOSUM62 矩阵进行，空位存在罚分为 11，且空位延伸罚分为 1；可与 SEQ ID NO:3 的序列进行最优比对而产生至少 e-74 的 e- 值得分的氨基酸序列，比对使用 BLOSUM62 矩阵进行，空位存在罚分为 11，且空位延伸罚分为 1；可与 SEQ ID NO:4 的序列进行最优比对而产生至少 210 的相似性得分的氨基酸序列，比对使用 BLOSUM62 矩阵进行，空位存在罚分为 11，且空位延伸罚分为 1；可与 SEQ ID NO:4 的序列进行最优比对而产生至少 e-66 的 e- 值得分的氨基酸序列，比对使用 BLOSUM62 矩阵进行，空位存在罚分为 11，且空位延伸罚分为 1；可与 SEQ ID NO:5 的序列进行最优比对而产生至少 170 的相似性得分的氨基酸序列，比对使用 BLOSUM62 矩阵进行，空位存在罚分为 11，且空位延伸罚分为 1；或者，可与 SEQ ID NO:5 的序列进行最优比对而产生至少 e-52 的 e- 值得分的氨基酸序列，比对使用 BLOSUM62 矩阵进行，空位存在罚分为 11，且空位延伸罚

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分为 1。

[0049] 在具体的实施方案中，所述分离的 Wnt7a 多肽还包含一个或多个 C 端氨基酸的 C 端缺失。

[0050] 在某些实施方案中，所述分离的 Wnt7a 多肽还包含至少 10 个 C 端氨基酸的 C 端缺失。

[0051] 在其他实施方案中，所述分离的 Wnt7a 多肽还包含至少 20 个 C 端氨基酸的 C 端缺失。

[0052] 在其他的实施方案中，所述分离的 Wnt7a 多肽包含具有生物活性的 Wnt7a 多肽或保留了 Wnt7a 生物活性。

[0053] 在一个实施方案中，根据本文公开的实施方案中任一个方案的分离的 Wnt7a 多肽保留了非经典的 Wnt7a 信号转导活性。

[0054] 在一个具体实施方案中，相比天然存在的 Wnt7a 多肽，根据本文所公开的实施方案中任一个方案的分离的 Wnt7a 多肽具有改良的产品收率。

[0055] 在某个实施方案中，相比天然存在的 Wnt7a 多肽，根据本文所公开的实施方案中任一个方案的分离的 Wnt7a 多肽具有改良的分泌特性。

[0056] 在又一个实施方案中，相比天然存在的 Wnt7a 多肽，根据本文所公开的实施方案中任一个方案的分离的 Wnt7a 多肽具有改良的稳定性或半衰期。

[0057] 在一个实施方案中，所述分离的 Wnt7a 多肽包含 SEQ ID NO:3-5 中任一序列所示的氨基酸序列。

[0058] 在一个具体实施方案中，所述分离的 Wnt7a 多肽包含 SEQ ID NO:3 所示氨基酸序列的至少 60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128 或 129 个连续氨基酸。

[0059] 在某个实施方案中，相比具有 SEQ ID NO:2 和 18-23 中任一序列所示氨基酸序列的 Wnt 多肽，所述分离的 Wnt7a 多肽具有增加的溶于水溶液的溶解度。

[0060] 在某个具体的实施方案中，所述分离的 Wnt7a 多肽结合细胞表面上的卷曲受体。

[0061] 在又一个具体实施方案中，所述细胞为骨骼肌卫星干细胞。

[0062] 在另一个实施方案中，相比缺少所述分离的 Wnt7a 多肽下的卫星干细胞扩增，所述分离的 Wnt7a 多肽与卷曲受体的结合增加了卫星干细胞扩增。

[0063] 在又一个实施方案中，Wnt7a 融合多肽包含根据本文所公开的实施方案中任一个方案的分离的 Wnt7a 多肽。

[0064] 在一个实施方案中，根据本文所公开的实施方案中任一个方案的 Wnt7a 融合多肽包含 Fc- 结构域。

[0065] 在另一个实施方案中，Wnt7a 融合多肽不具有 ADCC 或 CDC 活性。

[0066] 在某个实施方案中，相比天然存在的 Wnt7a 多肽，Wnt7a 融合多肽具有改良的产品收率。

[0067] 在一个具体实施方案中，相比天然存在的 Wnt7a 多肽，Wnt7a 融合多肽具有改良的分泌特性。

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[0068] 在另一个实施方案中，相比天然存在的 Wnt7a 多肽，Wnt7a 融合多肽具有改良的稳定性或半衰期。

[0069] 在一些实施方案中，所述 Wnt7a 融合多肽包含天然信号肽、异源信号肽，或天然和异源信号肽的杂合体。

[0070] 在一个实施方案中，所述 Wnt7a 融合多肽包含选自以下的异源信号肽：CD33 信号肽、免疫球蛋白信号肽、生长激素信号肽、红细胞生成素信号肽、白蛋白信号肽、分泌型碱性磷酸酶信号肽和病毒信号肽。

[0071] 在一个具体实施方案中，所述异源信号肽为 CD33 信号肽、IgGκ 信号肽或 IgGμ 信号肽。

[0072] 在另一个实施方案中，所述 Wnt7a 融合多肽包含异源蛋白酶切割位点。

[0073] 在又一个具体实施方案中，所述 Wnt7a 融合多肽包含选自以下的异源蛋白酶切割位点：烟草蚀纹病毒 (TEV) 蛋白酶切割位点、肝素切割位点、凝血酶切割位点、肠激酶切割位点和 Xa 因子切割位点。

[0074] 在另外的某个实施方案中，所述 Wnt7a 融合多肽包含选自以下的表位标签：HIS6 表位、MYC 表位、FLAG 表位、V5 表位、VSV-G 表位和 HA 表位。

[0075] 在又一个实施方案中，本发明提供了这样的多核苷酸，其编码根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽或 Wnt7a 融合多肽。

[0076] 在某个实施方案中，本发明提供了这样的载体，其包含编码根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽或 Wnt7a 融合多肽的多核苷酸。

[0077] 在某个具体的实施方案中，本发明提供了包含载体的宿主细胞，所述载体包含编码根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽或 Wnt7a 融合多肽的多核苷酸。

[0078] 在又一个实施方案中，所述宿主细胞为哺乳动物细胞、昆虫细胞或细菌细胞。

[0079] 在另一个实施方案中，根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽或 Wnt7a 融合多肽由所述宿主细胞产生。

[0080] 在一个实施方案中，本发明部分地包括：一种组合物，其包含根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽，或根据本文所公开的实施方案中任一个方案的 Wnt7a 融合多肽；一种多核苷酸，其编码根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽，或根据本文所公开的实施方案中任一个方案的 Wnt7a 融合多肽；或者，一种载体，所述载体包含编码根据本文所公开的实施方案中任一个方案的 Wnt7a 多肽或根据本文所公开的实施方案中任一个方案的 Wnt7a 融合多肽的多核苷酸。

[0081] 在一个具体实施方案中，所述组合物包含药学上可接受的盐、载体或赋形剂。

[0082] 在某个实施方案中，所述组合物可溶于水溶液。

[0083] 在又一个实施方案中，所述组合物被配制用于注射。

[0084] 在另一个实施方案中，所述组合物被配制用于以下的一种或多种：静脉内注射、心脏内注射、皮下注射、腹腔内注射或直接注射至肌肉。

[0085] 在各种实施方案中，所述组合物促进了组织形成、再生、维持或修复。

[0086] 在具体的实施方案中，所述组织为肌肉。

[0087] 在相关的实施方案中，所述肌肉为骨骼肌、心肌或平滑肌。

[0088] 在一个具体实施方案中，所述组合物促进了干细胞扩增。

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[0089] 在另一个实施方案中，所述干细胞为成体干细胞。

[0090] 在某个实施方案中，所述成体干细胞为卫星干细胞。

[0091] 在某个另外的实施方案中，所述组合物促进肌肉肥大或预防肌肉萎缩。

[0092] 在一个实施方案中，本发明部分地包括治疗或预防肌肉减少的方法，其包括将以下物质给予至对象：根据本文所公开的实施方案中任一个方案的截短的 Wnt 多肽、包含编码根据本文所公开的实施方案中任一个方案的截短的 Wnt 多肽的多核苷酸的载体，或者根据本文所公开的实施方案中任一个方案的组合物。

[0093] 在一个实施方案中，所述赋形剂增加了所述组合物的 Wnt7a 多肽或 Wnt7a 融合多肽的半衰期。

[0094] 在另一个实施方案中，所述赋形剂增加了所述组合物的 Wnt7a 多肽或 Wnt7a 融合多肽的稳定性。在一个具体实施方案中，所述组合物可溶于水溶液。

[0095] 在又一个实施方案中，所述组合物被配制用于注射。

[0096] 在一个实施方案中，所述组合物被配制用于以下的一种或多种：静脉内注射、心脏内注射、皮下注射、腹腔内注射或直接注射至肌肉。

[0097] 在另一个实施方案中，所述对象患有影响肌肉的疾病或病症，或者有患影响肌肉的疾病或病症的风险。

[0098] 在一个具体实施方案中，所述疾病为退行性疾病。

[0099] 在另一个具体实施方案中，所述退行性疾病为肌肉萎缩症。

[0100] 在某个具体的实施方案中，所述肌肉萎缩症选自：杜氏肌肉萎缩症 (DMD)、贝克氏肌肉萎缩症 (BMD)、埃 - 德二氏肌肉萎缩症、兰 - 迪二氏肌肉萎缩症、面肩胛臂肌肉萎缩症 (FSH)、肢 - 带型肌肉萎缩症、冯 •格雷菲 - 福克斯 (von Graefe-Fuchs) 肌肉萎缩症、眼咽型肌肉萎缩症 (OPMD)、强直性肌肉萎缩症（斯坦纳特病）和先天性肌肉萎缩症。

[0101] 在又一个具体实施方案中，所述影响肌肉的疾病或病症为消耗性疾病、肌肉衰减、肌肉萎缩、ICU 引起的虚弱、长期废用性肌萎缩 (prolonged disuse)、手术引起的虚弱或肌肉退行性疾病。

[0102] 在又一个具体实施方案中，所述病症是与肌肉废用、长期固定不动 (immobilization)、手术引起的虚弱或损伤有关的肌肉萎缩症。

[0103] 在又一个实施方案中，给予所述组合物可预防肌肉萎缩。

[0104] 在某个实施方案中，给予所述组合物促进了肌肉肥大。

[0105] 在一个具体实施方案中，所述肌肉为骨骼肌或心肌。

[0106] 在一个实施方案中，给予所述组合物促进了卫星干细胞扩增。

附图说明

[0107] 图 1 所示为人 Wnt 多肽和果蝇 (Drosophila) 多肽 WntD 的多重比对。

[0108] 图 2 所示为 Wnt7a 和 Wnt3a 的二级结构预测。A) 展示了带有编号 (10X) 的野生型人 Wnt7a 的氨基酸序列。显示了 ProteinPredict 二级结构预测软件结果 (Prof sec)，其中 H= α 螺旋，E= 折叠，且 L= 环或当无法确定确切结构时为空白。显示了每个预测的相对置信度分数 (Rel Sec)，并列出了 85% 以上置信度的区域 (SUB sec)。B) 展示了带有编号 (10X) 的野生型人 Wnt3a 的氨基酸序列。显示了 ProteinPredict 二级结构预测软件结果，

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其中 H= α 螺旋，E= 折叠，且 L= 环或当无法确定确切结构时为空白。显示了每个预测的相对置信度分数 (Rel Sec)，并列出了 85% 以上置信度的区域 (SUB sec)。

[0109] 图 3 所示为构建具有优选的药物特性的 Wnt 蛋白。示意图为按实施例 1 中所述设计和构建 Wnt7a 蛋白。除了野生型蛋白 (wtWnt7a) 外，所有蛋白中的信号肽都被突出显示为外源性的。在文本中描述了经设计以产生去脂化蛋白形式的点突变，并在示意图中将其突出显示。显示了截短物和 FC- 融合物。

[0110] 图 4 所示为各种 Wnt7a 蛋白形式的表达产率。按实施例 4 中所述表达了 Wnt7a 蛋白形式。显示了 Wnt 蛋白修饰和每升哺乳动物细胞培养基的 Wnt 蛋白产率。

[0111] 图 5 所示为各种纯化的 Wnt7a 蛋白形式的考马斯染色 SDS-PAGE 凝胶。按实施例 4 中所述表达并纯化了蛋白。将 500ng 的各 Wnt7a 蛋白加样至各泳道 :1) 可从 R&D systems 商购的 Wnt7a ;2) 将内源信号序列替换为 IgG κ - 链信号序列而表达和分泌的全长 Wnt7a ;3) 用 IgG κ 信号序列突变、去脂化的 Wnt7a ;4) 表达并纯化为 Fc- 融合蛋白的去脂化 Wnt7a ;5) 表达并纯化为 Fc- 融合蛋白的 Wnt7a 氨基酸 264-349 ;6) 表达并纯化为 Fc- 融合蛋白的 Wnt7a 氨基酸 264-349，其中 TEV 蛋白酶位点位于 Wnt 和 Fc 结构域之间，随后经蛋白水解消化并经色谱法净化以制备纯化的 Wnt7a 氨基酸 264-249。以最左侧泳道中的标记显示分子量。

[0112] 图 6 所示为 Wnt7a 在体外诱导了肌纤维肥大。如实施例 5 中所述，C2C12 小鼠成肌细胞分化为肌纤维并经 Wnt7a 蛋白处理。以制剂对照 (添加 1%CHAPS 的磷酸盐缓冲盐水) 与 wt Wnt7a 对比。

[0113] 图 7 所示为 Wnt7a 蛋白形式的体外肌纤维肥大数据。如实施例 5 中所述，C2C12 小鼠成肌细胞分化为肌纤维并经 Wnt7a 蛋白处理。对纤维直径进行定量，并显示为来自 200 个测量值的平均纤维直径。将制剂对照 (添加 1%CHAPS 的磷酸盐缓冲盐水) 与 wt Wnt7a、wtWnt7a-Fc- 融合蛋白或 Wnt7a264-349-Fc- 融合蛋白对比。在 CHAPS 清洁剂存在和不存在的情况下测量所有 Wnt 蛋白。

[0114] 图 8 所示为 Wnt7a 蛋白形式的体外肌纤维肥大数据。如实施例 5 中所述，C2C12 小鼠成肌细胞或原代人抗肌萎缩病 (dystrophinopathy) 成肌细胞分化为肌纤维并经 Wnt7a 蛋白处理。对纤维直径进行定量，并显示为来自 100 个测量值的平均纤维直径。A) 制剂对照 (添加 1%CHAPS 的磷酸盐缓冲盐水 (PBSC)) 与 wt Wnt7a 和小鼠 C2C12 肌纤维的截短的 Wnt7a 氨基酸 235-349 比较。B) 将制剂对照 (添加 1%CHAPS 的磷酸盐缓冲盐水 (PBSC)) 与 wt Wnt7a 和人抗肌萎缩病肌纤维的截短的 Wnt7a 氨基酸 235-349 比较。C) 截短的 Wnt7a 氨基酸 264-349 的剂量反应。

[0115] 图 9 所示为各种 Wnt7a 蛋白的加速的蛋白稳定性评估的定量。各种 Wnt7a 蛋白形式——(A)wtWnt7a-FC、(B)Wnt7a aa264-349-FC 和 (C)Wnt7a264-349 以相同的蛋白浓度于 4℃或 37℃孵育 0、1、4 或 7 天。评估了 3 种不同的赋形剂制剂 :0.2%CHAPS/PBS、0.05% 聚山梨醇酯 80(PS80) 或仅 PBS。用蛋白印迹分析评估剩余的蛋白，然后用像素密度测定法将其转换为剩余蛋白部分相比起始量 (时间为 0) 的值。

[0116] 图 10 所示为来自加速的稳定性研究的 Wnt7a 样品的肌纤维肥大评估。各种 Wnt7a 蛋白形式以相同的蛋白浓度于 4℃或 37℃孵育 0、1、4 或 7 天。评估了赋形剂制剂 0.2%CHAPS/PBS。如实施例 5 和 6 中所述，在体外肌纤维肥大测定中评估了剩余蛋白的活

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性。使用了阴性制剂对照和阳性的可市购的 Wnt7a 蛋白对照。将 A) Wnt7a 和 Wnt7a-Fc- 融合物，以及 B) 截短的 Wnt7a264-349 和截短的 Wnt7a264-349-Fc- 融合蛋白进行比较。

[0117] 图 11 所示为来自加速的稳定性研究的 Wnt7a 样品的肌纤维肥大评估。各种 Wnt7a 蛋白形式以相同的蛋白浓度于 4℃或 37℃孵育 0、1、4 或 7 天。评估了赋形剂制剂 0.05% 聚山梨醇酯 80（吐温）/PBS。如实施例 5 和 6 中所述，在体外肌纤维肥大测定中评估了剩余蛋白的活性。使用阴性制剂对照和阳性的可市购的 Wnt7a 蛋白对照。将 A) Wnt7a 和 Wnt7a-Fc- 融合物，以及 B) 截短的 Wnt7a264-349 和截短的 Wnt7a264-349-Fc- 融合蛋白进行比较。

[0118] 图 12 显示 Wnt7a 蛋白形式并非经典 Wnt 信号转导通路的激活剂。将 pBAR 经典 Wnt 报告系统引入至来自不同组织的四个细胞系中。测定以下每个系对 Wnt 信号转导的应答 :A) A549 系、B) KG-1a 系、C) NALM-6 系，和 D) TF-1a 系。Wnt3a 在所有测定的系中均产生了清楚的荧光素酶报告子应答。全长 Wnt7a(FTV500) 和截短的 Wnt7a aa264-349-Fc 融合物 (FTV526) 没有在任何测定浓度下诱导经典的 Wnt 报告子。

[0119] 图 13 显示 Wnt7a 在体内诱导了明显的肥大。就其单次注射至 C57B16 小鼠胫骨前肌后诱导肥大的能力，将全长 Wnt7a 与制剂对照或 IGF-1 进行比较。A) 层粘连蛋白的免疫组织化学染色显示了用制剂对照或 Wnt7a 处理的肌肉中的纤维横切面。B) 针对每个处理组，从每动物 1000 个值计算了纤维菲雷特 (Feret) 直径的中值，并绘制了以下每个处理组的中值的动物间均值 :对侧的（未处理的）对照、制剂对照、IGF-L 或 Wnt7a。C) 所有的纤维菲雷特直径 / 处理组均作为群体分析进行标绘，其中标绘了中值和四分位值。D) 针对中值、均值和统计显著性 (*p ≤ 0.05) 的处理组之间的值。

[0120] 图 14 显示了 Wnt7a aa264-349 在体内诱导了明显的肥大。就其单次注射至 MDX 抗肌萎缩病小鼠模型的胫骨前肌后诱导肥大的能力，将 Wnt7a aa264-349-Fc- 融合蛋白与 Fc- 融合物对照进行比较。A) 从每动物 1000 个值计算了纤维菲雷特直径的中值，并绘制了以下每个处理组的中值的动物间均值 :对侧的（未处理的）对照、Fc- 融合物对照或用或不用 CHAPS 清洁剂配制的 Wnt7a264-349-Fc- 融合蛋白。B) 所有纤维菲雷特直径 / 处理组均作为群体分析进行标绘，其中标绘了中值和四分位值。C) 针对中值、均值和统计显著性 (*p ≤ 0.05) 的处理组之间的值。

[0121] 图 15 显示了 Wnt7a 蛋白形式的药代动力学分析。于 C57B16 小鼠中，在单次静脉内给药 PK 研究中评估了各种 Wnt7a 蛋白形式。比较了全长 Wnt7a(FTV500)、Wnt7a-Fc- 融合物 (FTV512)、Wnt7a aa264-349 片段 (FTV529) 和 Wnt7a aa264-349-Fc- 融合蛋白 (FTV526)。A) 绘制了所示时间点的对小鼠血浆的 Wnt7a 特异性 ELISA。B) 显示了排除 FTV526 后的展开的的数据集。

[0122] 序列标识说明

[0123] SEQ ID NO:1 所示为人 Wnt7a 的 cDNA 序列。

[0124] SEQ ID NO:2 所示为由 SEQ ID NO:1 编码的人 Wnt7a 多肽的氨基酸序列。

[0125] SEQ ID NO:3 所示为 SEQ ID NO:2 的氨基酸 221-349。

[0126] SEQ ID NO:4 所示为 SEQ ID NO:2 的氨基酸 235-349。

[0127] SEQ ID NO:5 所示为 SEQ ID NO:2 的氨基酸 264-349。

[0128] SEQ ID NO:6-9 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:3 的氨基酸序列。

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[0129] SEQ ID NO:10-13 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:4 的氨基酸序列。

[0130] SEQ ID NO:14-17 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:5 的氨基酸序列。

[0131] SEQ ID NO:18 所示为小鼠 Wnt7a 多肽的氨基酸序列。

[0132] SEQ ID NO:19 所示为大鼠 Wnt7a 多肽的氨基酸序列。

[0133] SEQ ID NO:20 所示为鸡 Wnt7a 多肽的氨基酸序列。

[0134] SEQ ID NO:21 所示为斑马鱼 Wnt7a 多肽的氨基酸序列。

[0135] SEQ ID NO:22 所示为猪 Wnt7a 多肽的氨基酸序列。

[0136] SEQ ID NO:23 所示为牛 Wnt7a 多肽的氨基酸序列。

[0137] SEQ ID NO:24-26 所示为用于构建 Wnt 表达载体的多核苷酸序列。

[0138] SEQ ID NO:27 所示为编码 CD33 信号肽的多核苷酸序列。

[0139] SEQ ID NO:28 所示为由 SEQ ID NO:27 的多核苷酸序列编码的氨基酸序列。

[0140] SEQ ID NO:29 所示为编码 IgGκ 信号肽的多核苷酸序列。

[0141] SEQ ID NO:30 所示为由 SEQ ID NO:29 的多核苷酸序列编码的氨基酸序列。

[0142] SEQ ID NO:31-32 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:3 的氨基酸序列。

[0143] SEQ ID NO:33-34 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:4 的氨基酸序列。

[0144] SEQ ID NO:35-36 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:5 的氨基酸序列。

[0145] SEQ ID NO:37-38 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:4 的氨基酸序列。

[0146] SEQ ID NO:39-40 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:5 的氨基酸序列。

[0147] SEQ ID NO:41-42 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:4 的氨基酸序列。

[0148] SEQ ID NO:43-44 所示为融合多肽的氨基酸序列，所述融合多肽包含 SEQ ID NO:5 的氨基酸序列。

[0149] 发明详述

[0150] A. 综述

[0151] 虽然 Wnt 的翻译后脂化被认为是生物活性和蛋白分泌所必需的，本发明仍部分地提供了新的截短的具有 Wnt 生物活性的 Wnt 多肽，包括缺少一个或多个脂化位点的截短形式的 Wnt。本发明的多肽保留了 Wnt 生物活性，因此本发明提供了修饰的 Wnt 多肽和包含其的组合物以及此类 Wnt 多肽的治疗应用，所述 Wnt 多肽或组合物具有改良的类生物药特性，如改良的溶解度、生产、剂型、全身性递送和组织吸收。针对由 Wnt 多肽的不溶性引起的问题，本发明还提供了新的解决方案，且进一步提供了独创性的 Wnt 多肽，其适于临床规模上的生产和治疗应用。本发明 Wnt 多肽的治疗应用包括，例如，促进干细胞扩增、组织形成，以及细胞和 / 或组织再生、修复或维持。

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[0152] 除非另有明确的说明，本发明的实施可利用本技术领域中以下方面的常规方法：化学、生物化学、有机化学、分子生物学、微生物学、重组 DNA 技术、遗传学、免疫学和细胞学，为说明的目的其中许多在下文有描述。这类技术在文献中有完整的说明。参见，例如，Sambrook, et al., Molecular Cloning:A Laboratory Manual(3rd Edition, 2001);Sambrook, et al., Molecular Cloning:A Laboratory Manual(2nd Edition, 1989);Maniatis et al., Molecular Cloning:A Laboratory Manual(1982);Ausubel et al., Current Protocols in Molecular Biology(John Wiley and Sons, 2008 年 7 月 更 新);Short Protocols in Molecular Biology:A Compendium of Methods from Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience;Glover, DNA Cloning:A Practical Approach, vol. I&II(IRL Press, Oxford, 1985);Anand, Techniques for the Analysis of Complex Genomes, (Academic Press, New York, 1992);Transcription and Translation(B. Hames&S. Higgins, Eds., 1984);Perbal, A Practical Guide to Molecular Cloning(1984)； 以 及 Harlow and Lane, Antibodies, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1998)。

[0153] 本文引用的所有出版物、专利和专利申请在此均通过引用整体并入本文。

[0154] B. 定义

[0155] 除非另有说明，本发明所用的所有技术和科学术语具有的含义与本发明所属领域技术人员通常所理解的相同。尽管在本发明的实施或测试中，可使用任何类似或等同于本文所述的那些的方法和材料，本文描述了组合物、方法和材料的优选实施方案。为本发明的目的对下列术语定义如下。

[0156] 本文所用冠词是指一个 / 种或多于一个 / 种（即，至少一个 / 种）的所述冠词的语法客体。例如，“元件”是指一个或多于一个的元件。

[0157] 如本文所用，术语“约 (about/approximately)”是指相对于参考的数量、水平、值、数目、频率、百分比、尺寸、大小、量、重量或长度，数量、水平、值、数目、频率、百分比、尺寸、大小、量、重量或长度变化差不多为 30%、25%、20%、25%、10%、9%、8%、7%、6%、5%、4%、3%、2% 或 1%。在具体的实施方案中，术语“约 (about/approximately)”位于数值前时表示该数值加或减 15%、10%、5% 或 1% 的范围。

[0158] 在本说明书中提及“一个实施方案”、“一实施方案”、“一个具体实施方案”、“一个相关实施方案”、“某个实施方案”、“另一个实施方案”或“又一个实施方案”或其组合是指描述的与该实施方案相关的具体特征、结构或特性包含于本发明的至少一个实施方案中。因此，在整个本说明书的不同地方出现上述词语，并不一定都指相同的实施方案。而且，所述具体特征、结构或特性能以任何合适的方式组合于一个或多个实施方案中。

[0159] 如本文所用，术语“干细胞”是指未分化的细胞，其能够 :(1) 长期自我更新，或能够产生原始细胞的至少一个相同拷贝，(2) 在单细胞水平上分化为多种且在某些情况下仅为一种特化的细胞类型，以及 (3) 在体内进行组织的功能性再生。根据它们的发展潜力，将干细胞再分类为 :全能干细胞 (totipotent)、多能干细胞 (pluripotent)、专能干细胞 (multipotent) 和寡能 / 单能干细胞 (oligo/unipotent)。

[0160] 如本文所用，术语“成体干细胞 (adult stem cell/somatic stem cell)”是指在发

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育后或发育中的生物体中所发现的干细胞；通常在生物体的特定组织中。可通过细胞分裂进行分裂的成体干细胞为专能干细胞或单能干细胞，其随后进行分化以增加、替代或再生失去的细胞和/或组织。成体干细胞包括但不限于，外胚层干细胞、内胚层干细胞、中胚层干细胞、神经干细胞、造血干细胞、肌肉干细胞、卫星干细胞等。肌肉干细胞是常规上被认为属于单能干细胞的干细胞的实例，其仅产生肌肉细胞。

[0161] 如本文所用，术语“卫星干细胞”是指产生肌原性细胞谱系例如成肌细胞和肌细胞的成体干细胞类型。在一个实施方案中，所述卫星干细胞为 $Pax7^{+}/Myf5^{-}$ 肌肉干细胞。在一个具体实施方案中，所述卫星干细胞为骨骼肌干细胞。

[0162] 如本文所用，术语“祖细胞”是指能够进行自我更新并分化为更成熟细胞的细胞，但其定向（commit）于一种谱系（例如，造血祖细胞定向于血液谱系），然而干细胞不一定受此限制。成肌细胞为祖细胞的实例，其仅能够分化为一种细胞类型，但其本身没有完全成熟或完全分化。成肌细胞可分化为肌细胞。

[0163] 如本文所用，术语“肌细胞”或“肌纤维”是指肌肉中发现的分化细胞类型。每个肌细胞含有肌原纤维即肌节长链，其为肌肉细胞的收缩单元。有各种特定形式的肌细胞：心肌细胞、骨骼肌细胞和平滑肌细胞，其具有本领域已知的各种性能。

[0164] 如本文所用，术语“自我更新”是指具有产生不变的子细胞以及产生特化细胞类型（潜力）的独特能力的细胞。自我更新能以至少两种方式实现。不对称的细胞分裂产生一个与母细胞相同的子细胞和一个与母细胞不同的子细胞，其为祖细胞或分化细胞。因此，不对称的细胞分裂不增加与母细胞相同的子细胞的数目，但维持母细胞类型的细胞数目。反之，对称的细胞分裂产生两个各自与母细胞相同的子细胞。因此，对称的细胞分裂增加了与母细胞相同的细胞的数目，扩增了母细胞群体。在具体的实施方案中，对称的细胞分裂和细胞扩增例如干细胞群体扩增可互换使用。

[0165] 如本文所用，术语“分化”是指细胞借以特化为具有特定功能的发育过程，例如，其中细胞获得了不同于原始细胞类型的一种或多种形态特征和/或功能。术语“分化”包括谱系定向和末期分化过程。例如，可通过利用本领域技术人员已知的免疫组织化学或其他方法评估或监测生物标记的存在或不存在，评估未分化或分化状态。

[0166] 如本文所用，术语“谱系定向”是指干细胞借以定向为形成具体限定范围的分化细胞类型的过程。例如，当干细胞在不对称细胞分裂期间产生了祖细胞时，谱系定向就发生了。定向的祖细胞通常能够自我更新或进行细胞分裂。

[0167] 如本文所用，术语“末期分化”是指细胞最后分化为成熟细胞，即完全分化的细胞。通常末期分化与退出细胞周期和停止增殖有关。

[0168] 如本文所用，术语“肌肉肥大”是指肌肉大小的增加，并且可包括单根纤维体积的增加和/或肌纤维横截面积的增加，并且还可包括每根肌纤维中细胞核数目的增加。肌肉肥大也可包括整个肌肉的体积和质量的增加；然而，肌肉肥大可能分化自肌肉增生，其形成新肌肉细胞。在一个实施方案中，肌肉肥大是指肌动蛋白和肌球蛋白收缩蛋白的数目增加。

[0169] 如本文所用，术语“促进”、“增强”、“刺激”或“增加”通常是指相比由媒介物（vehicle）或对照分子/组合物引起的响应，本发明的 Wnt 多肽或组合物能够产生或引起更大的生理响应（即可测量的下游效应）。一种这类可测量的生理响应包括但不限于，相比不对称的细胞分裂，对称的干细胞分裂的增加，例如，相比正常的、未处理的或对照处理

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的肌肉细胞，卫星干细胞的增加和 / 或肌肉肥大的增加。相比天然下发现的 Wnt 多肽，本发明的 Wnt 多肽和组合物也可具有“改良的”、“增加的”、“增强的”或“更大的”物理和药代动力学特性。例如，本发明 Wnt 多肽的生理响应、物理特性或药代动力学特性可增加至少 5%、10%、20%、30%、40%、50%、60%、70%、80%、90%、100%、125%、150%、175%、200% 或更大。在另一个非限制性实例中，相比天然 Wnt，本发明 Wnt 组合物的独创性 Wnt 多肽的生理响应、物理特性或药代动力学特性可增加至少 5%、10%、20%、30%、40%、50%、60%、70%、80%、90%、100%、125%、150%、175%、200% 或更大。“增加的”或“增强的”响应或特性通常为“具有统计学意义的”，并且可包括这样的增加，其相比媒介物（无药剂）或对照 Wnt 组合物，为 1.1、1.2、1.5、2、3、4、5、6、7、8、9、10、15、20、30 倍或更多倍（例如 500、1000 倍）（包含其间的且大于 1 的所有整数和小数值，例如 1.5、1.6、1.7、1.8 等）。

[0170] 如本文所用，术语“保留”、“维持”通常是指本发明的 Wnt 组合物能够产生或引起生理响应（即可测量的下游效应），其与天然存在的 Wnt 氨基酸或核苷酸序列的 Wnt 组合物所产生的响应特性类似。例如，本发明的 Wnt 组合物展现了 Wnt 生物活性，并因此保留了 Wnt 活性。本发明的组合物还能产生生理响应如肌肉肥大，其与天然存在的 Wnt 多肽所产生的响应特性类似。本发明的产生类似生理响应的 Wnt 组合物可产生的生理响应为包含天然存在的 Wnt 氨基酸或核苷酸序列的组合物所产生的生理响应水平的至少 5%、至少 10%、至少 15%、至少 20%、至少 25%、至少 30%、至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95% 或约 100%。

[0171] 如本文所用，术语“减少”或“降低”或“减轻”或“减小”或“缩减”通常是指相比媒介物或对照分子 / 组合物所产生的响应，本发明 Wnt 组合物能够产生或引起更小的生理响应（即下游效应），例如降低的细胞凋亡。在一个实施方案中，所述降低可为基因表达的降低或通常与细胞活性降低有关的细胞信号转导的降低。“降低”或“减小”的响应通常为“具有统计学意义的”响应，并且可包括这样的降低，其相比媒介物（无药剂）或对照组合物，所产生的响应降低 1.1、1.2、1.5、2、3、4、5、6、7、8、9、10、15、20、30 倍或更多倍（例如 500、1000 倍）（包含其间的且大于 1 的所有整数和小数值，例如 1.5、1.6、1.7、1.8 等）。

[0172] C. Wnt 信号转导通路

[0173] Wnt 信号转导通路是古老且进化上保守的通路，其在发育期间和成体整个一生中调控细胞命运决定、细胞迁移、细胞极性、神经模式形成和器官发生的关键方面。Fzd 受体下游的 Wnt 信号转导通路已被确认包括经典的或 Wnt/β-连环蛋白依赖性通路以及非经典的或 β-连环蛋白非依赖性通路，其可进一步分为平面细胞极性通路、Wnt/Ca^{2+} 通路等。

[0174] Wnt 蛋白结合至卷曲 (Fzd) 受体家族的 N-端胞外半胱氨酸富集结构域。在人体中有 10 个 Fzd 受体。Fzd 蛋白是与 G- 蛋白偶联受体具有拓扑学同源性的 7 次跨膜蛋白。此外，对于 Wnt 和 Fzd 之间的相互作用，还需要共受体来介导 Wnt 信号转导。例如需要低密度脂蛋白相关的蛋白 5/6(LRP5/6) 来介导经典的 Wnt 信号，而受体酪氨酸激酶 RYK 可能是非经典功能所必需的。另一水平上的 Wnt 信号转导调控在存在不同数量的分泌的 Wnt 拮抗剂下发生于胞外环境中。Wnt 结合至受体复合体后，信号被转导至胞质磷蛋白蓬乱蛋白 (Dsh/Dvl)。Dsh 能直接与 Fzd 相互作用。在 Dsh 水平上，所述 Wnt 信号分支成 3 个主要级联——经典的（β-连环蛋白）、平面细胞极性和 Wnt/Ca^{2+}。而且，G- 蛋白偶联受体信号转导也可刺激生长和存活通路如 PI3K。

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[0175] 1. 经典的 Wnt 信号转导通路

[0176] 从果蝇 (Drosophila) 的遗传筛选中首先确认并描述了经典的 Wnt 信号转导通路，且对苍蝇、蠕虫、青蛙、鱼和小鼠中的深入研究已使得确认了基本的分子信号转导框架。经典的 Wnt 通路的标志为粘合连接相关蛋白 β－连环蛋白的积累并转移至细胞核。在无 Wnt 信号转导的情况下，胞质的 β－连环蛋白被 β－连环蛋白分解复合体降解，所述复合体包含轴蛋白 (Axin)、腺瘤性结肠息肉 (APC) 蛋白、蛋白磷酸酶 2A(PP2A)、糖原合成酶激酶 3β (GSK3β) 和酪蛋白激酶 1α (CK1α)。该复合体内的 β－连环蛋白通过 CK1α 和 GSK3β 磷酸化将其作为目标，用于泛素化和随后的通过蛋白酶体机制进行的蛋白水解分解。Wnt 结合至其由 Fzd 和 LRP5/6 组成的受体复合体，通过 CK1 和 GSK3-β 诱导了 LRP6 的双磷酸化，这使得能够将含轴蛋白的蛋白复合体从胞液转移至细胞质膜。Dsh 也被募集至所述膜并结合至 Fzd，且轴蛋白结合至磷酸化的 LRP5/6。在膜上的 Fzd/LRP5/6 处形成的该复合体，通过轴蛋白的隔离和 / 或降解引起 β-cat 稳定。β－连环蛋白转移至细胞核中，在那里其与 Lef/Tcf 家族成员复合以介导靶基因的转录诱导。

[0177] 经典的 Wnt 信号转导影响前部的头结构的形成和神经外胚层模式发生、后部模式发生和尾形成，以及各种器官系统包括心、肺、肾、皮肤和骨等的形成。

[0178] 2. 非经典的 Wnt 信号转导通路

[0179] 非经典的通路通常被称为 β－连环蛋白非依赖性通路，但其不像经典的通路那样得到充分定义，该通路可进一步分为至少两个不同分支——平面细胞极性通路 (或 PCP 通路) 和 Wnt/Ca2+ 通路，其中本文仅详细论述 PCP。PCP 通路出现于对果蝇的遗传研究，其中发现 Wnt 信号转导元件包括卷曲蛋白和蓬乱蛋白的突变使上皮结构包括表皮毛发和感觉刚毛方向随机化。已知上皮细胞具有确定的顶部－基底侧极性，但此外它们还沿着上皮层的平面具有极性。该刚性组织决定了结构的方向，包括毛囊的方向、感觉刚毛和眼睛中六角形小眼排列的方向。在脊椎动物中，已表明该组织构成了肌肉细胞、内耳的感觉上皮的静纤毛、毛囊组织的组织和方向，以及经历原肠胚形成的背部中胚层细胞形态和迁移行为的基础。

[0180] Wnt 信号转导通过独立于 LRP5/6 的 Fzd 进行转导，其导致 Dsh 的激活。Dsh 通过 Daam1 介导 Rho 的激活，其转而激活 Rho 激酶 (ROCK)。Daam1 还通过结合肌动蛋白的前纤维蛋白 (Profilin) 介导肌动蛋白聚合。Dsh 还介导 Rac 的激活，其转而激活 JNK。在原肠胚形成期间，来自 Rock、JNK 和前纤维蛋白的信号被整合用于细胞骨架改变，以用于细胞极化和运动。

[0181] 3. 肌肉细胞发育中的 Wnt 信号转导

[0182] 卫星干细胞为产生肌肉细胞的成体干细胞。成体骨骼肌中的卫星细胞位于其宿主肌纤维的肌纤维膜和基膜之间的小凹陷中。在受到损伤时，如物理创伤、反复锻炼或生病时，卫星细胞被激活而增殖，并产生表达肌原性调控因子 (MRF)——MyoD 和 Myf5 的肌原性前体细胞 (成肌细胞) 群体。在再生的过程中，成肌细胞经历多轮分裂，然后定向于末期分化，与宿主纤维融合或产生新的肌纤维以重建受损组织 (Charge 和 Rudnicki, 2004)。在骨骼肌再生过程中，所述卫星干细胞群体通过干细胞亚群得以扩增和维持，从而允许组织的体内平衡和个体寿命期间的多轮再生 (Kuang et al., 2008)。卫星干细胞 (Pax7+/Myf5-) 代表约 10% 的成体卫星细胞库，并通过不对称的顶部－底部细胞分裂产生子代卫星肌原细胞

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(Pax7+/Myf5+)。

[0183] Wnt 信号转导在调控整个胚胎发育的发育程序和调控成体组织的干细胞功能中起着关键作用 (Clevers, 2006)。Wnt 对于轴旁中胚层的胚胎肌原性诱导 (Borello et al., 2006; Chen et al., 2005; Tajbakhsh et al., 1998) 以及肌肉纤维发育期间的分化控制 (Anakwe et al., 2003) 是必需的。最近,有人认为 Wnt 平面细胞极性 (PCP) 通路与肌节发育中调控肌细胞生长方向有关 (Gros et al., 2009)。在成体中,Wnt 信号转导被认为对于急性损伤后肌肉组织中成体干细胞的肌原性定向是必需的 (Polesskaya et al., 2003; Torrente et al., 2004)。其他研究表明,Wnt/β-连环蛋白信号转导通过激活和募集储备的成肌细胞来调控肌原性分化 (Rochat et al., 2004)。此外,成体肌肉中卫星细胞的 Wnt/β-连环蛋白信号转导似乎是通过限制 Notch 信号转导来控制肌原性谱系发展,从而促进分化 (Brack et al., 2008)。

[0184] 最近已确定,在静止的卫星干细胞中,Wnt 受体 Fzd7 被明显地上调。此外,进一步研究揭示,在肌肉再生期间,Wnt7a 被表达并通过其受体 Fzd7 和 Vangl2(平面细胞极性 (PCP) 通路的元件)发挥作用,从而诱导对称的卫星干细胞扩增并有力地促进肌肉再生。

[0185] 在 PCP 通路中抑制受体或效应分子例如 Fzd7 或 Vangl2 被认为消除了 Wnt7a 对卫星干细胞的影响 (Le Grand et al., 2009)。已经进一步证明,给予脂化的 Wnt7a 多肽或编码 Wnt7a 多肽(其随后通过脂化进行翻译后修饰)的多核苷酸,在体内和体外均显著地增加了卫星干细胞的数目,并促进了体内组织形成,导致损伤和患病肌肉组织中的修复和再生增加 (Le Grand et al., 2009)。

[0186] 不希望受到任何具体理论的束缚,预计导致促进损伤和患病肌肉组织中的修复和再生增加 Wnt7a 作用机制有两条途径 :Wnt7a 可通过 PCP 通路刺激肌肉卫星(干)细胞的对称扩增,从而产生随后能够分化为成肌细胞的较大细胞库 ;其次,通过 G- 蛋白偶联受体(卷曲蛋白),Wnt7a 可刺激成肌细胞和肌纤维中的磷脂酰肌醇 3- 激酶 /Akt(蛋白激酶 B)/ 哺乳动物雷帕霉素靶标 (PI3K/Akt/mTOR) 通路信号转导,其已被表明能刺激肥大 (Bodine et al., Nature Cell Biology. 2001; vol. 3; pp. 1014-1017; Glass et al., Nature Cell Biology. 2003; vol. 5; pp. 87-90; Ciciliot and Schiaffino, Current Pharmaceutical Design. 2010; 16(8); pp. 906-914)。Wnt7a 可通过 G- 蛋白偶联受体卷曲蛋白 7 发出信号,且该 Wnt/Frz 相互作用能有助于两种生物效应。

[0187] 在各种实施方案中,组合物包含修饰的 Wnt,尤其是 Wnt 融合多肽(例如,Fc- 融合物)或者缺少 N 端和 / 或 C 端氨基酸的截短的 Wnt 多肽。本发明的截短的 Wnt 多肽缺少一个或多个脂化位点,但仍然出人意料地保留了 Wnt 生物活性、受体结合特异性,并相比脂化的 Wnt 具有改良的溶解度、生产、全身性递送和组织吸收。在具体的实施方案中,本发明的组合物包含修饰的 Wnt7a,尤其是 Wnt7a 融合多肽或者缺少 N 端和 / 或 C 端氨基酸的截短的 Wnt7a 多肽,例如,缺少 N 端至少 220 个氨基酸的 Wnt7a 多肽。所述截短的 Wnt7a 多肽缺少一个或多个脂化位点,但仍然出人意料地保留了 Wnt7a 生物活性、受体结合特异性,并相比脂化的 Wnt 具有改良的溶解度、生产、全身性递送和组织吸收。

[0188] 尽管 PI3K/Akt/mTOR 通路对于肌肉细胞肥大的重要性已被描述,在肌肉细胞中特异性刺激该通路的治疗挑战,存在促进损伤的和患病的肌肉组织中的修复和再生的明显障碍。利用有效的 PI3- 激酶活化剂如 IGF-1 的早期研究在体外产生了肥大,但可能存在“脱

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靶”的代谢效应（即，IGF-1 和 PI3K 是看家性代谢、生存和代谢过程的关键调控因子）。因此，PI3K/Akt/mTOR 通路的肌肉特异性 Wnt7a-Fzd7 刺激可能性将代表重要和独特的治疗突破。

[0189] 如下文所进一步详细描述，本发明部分地包括独创性的 Wnt 多肽，其提供了针对该技术障碍以及应用 Wnt 多肽治疗来促进损伤和患病肌肉组织中的修复和再生的其他障碍的出人意料的解决方案。

[0190] D. 多肽

[0191] Wnt 信号转导通路是细胞信号转导网络的关键元件。人 Wnt 基因家族由 19 个成员组成，其以 22 个或 24 个 Cys 残基以及几个保守的 Asn 和 Ser 残基编码进化上保守的糖蛋白。示例性人 Wnt 蛋白包括 Wnt1、Wnt2、Wnt2b/13、Wnt3、Wnt3a、Wnt4、Wnt5a、Wnt5b、Wnt6、Wnt7a、Wnt7b、Wnt8、Wnt8a、Wnt8b、Wnt9a、Wnt9b、Wnt10a、Wnt10b、Wnt11 和 Wnt16。

[0192] 所述 Wnt 为分泌型糖蛋白，其在运输和释放至胞外环境前被大幅修饰。在信号序列切割和转移至内质网 (ER) 后，Wnt 通过内膜系统被运输至细胞表面并接受一些修饰。Wnt 接受了连接于 N 端的糖基化 (Burrus and McMahon1995;Kadowaki et al.,1996;Komekado et al.,2007;Kurayoshi et al.,2007;Mason et al.,1992;Smolich et al.,1993;Tanaka et al.2002)。许多 Wnt 还在第一保守半胱氨酸处被棕榈酰化，例如，Wnt1 的 C93、Wnt3a 的 C77 和 Wnt5a 的 C104(Galli et al.,2007;Kadowaki et al.,1996;Komekado et al.,2007;Willert et al.2003)。此外，Wnt3a 在保守丝氨酸 S209 处被棕榈油酸修饰，该保守丝氨酸在 Wnt1(S224)Wnt5a 中也是保守的 (Takada et al.,2006)。而且，这些保守的半胱氨酸和丝氨酸残基存在于许多 Wnt 的 N 端，例如，Wnt1、Wnt3a、Wnt4、Wnt5a、Wnt6、Wnt7a、Wnt9a、Wnt10a 和 Wnt11 等 (Takada et al.,2006)。

[0193] Wnt 酰化被广泛认为导致了分泌的 Wnt 众所周知的疏水性 (Willert etal.,2003)。此外，哺乳动物 Wnt 的翻译后脂化被认为对发挥功能非常重要。使 Wnt1、Wnt3a 或 Wnt5a 的保守 N 端半胱氨酸突变阻止了细胞培养中的棕榈酰化。这些突变的 Wnt 能分泌，但被表明具有极少或没有信号转导活性 (Galli et al.,2007;Komekado et al.,2007;Kurayoshi et al.,2007;Willert et al.,2003)，而未棕榈酰化的 Wnt 被认为无法结合 Fzd 受体 (Komekado et al.,2007;Kurayoshi et al.2007)。使 Wnt3a 中心部位的保守丝氨酸突变阻止了棕榈油酸的添加并阻碍了分泌，并由此阻碍了活性 (Takada et al.,2006)。对果蝇 Wg 的研究证明了酰化的重要性 (Franch-Marro et al.,2008a;Nusse2003;van den Heuvel et al.,1993)。

[0194] 此外，这些数据得到果蝇中豪猪 (porc) 表型的支持，其显示明显丧失了 Wg 信号转导 (van den Heuvel et al.,1993)。Porc 是位于 ER 的整合膜 O- 酰基转移酶 (Kadowaki et al.,1996)，其为 Wg 棕榈酰化 (Zhai et al.,2004) 和 Wg ER 排除 (Tanaka et al.,2002) 所必需。脊椎动物的 Porc 也促进了 Wnt 脂化，且是 Wnt 信号转导和 Wnt 生物活性所必需 (Galli et al.,2007)。

[0195] 这些研究建立了这样的模型，其中棕榈油酸修饰是分泌所必需，而棕榈酸酯是 Fzd 结合所必需。因此，缺少用于这些报道的脂质修饰之一或两者的 N 端氨基酸序列的 Wnt 多肽预计会缺少生物活性。

[0196] 在各种实施方案中，本发明部分地包括 Wnt 多肽例如，截短的 Wnt、Wnt 融合多肽，其保留了 Wnt 生物活性，但其经改造而去除了 WntN 端的翻译后修饰位点，所述位点对溶解

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度、生产、全身性递送和组织吸收有不良影响。在具体的实施方案中，本发明的 Wnt 多肽促进干细胞和祖细胞扩增以及肌肉肥大，并促进细胞和 / 或组织形成、再生、维持和修复。如本文所用，术语“经典的”是指天然存在多肽中存在的氨基酸或氨基酸基团。在一些情形中，当指天然存在多肽中存在的氨基酸时，“经典的”和“天然的”可互换使用。

[0197] 在某些实施方案中，Wnt 多肽为截短的，例如，缺少天然 Wnt 多肽的 N 端和 / 或 C 端氨基酸。在一些具体的实施方案中，所述 Wnt 多肽包含 N 端和 / 或 C 端截短，但保留了或具有增加的经典的和 / 或非经典的 Wnt 信号转导活性。

[0198] 如本文所用，除非另有说明，术语“多肽”、“肽”和“蛋白”可互换使用，并依照常规含义，即作为由肽键或修饰的肽键连接的氨基酸序列。如本文其他地方所述，本发明的多肽包括但不限于，截短的多肽、具有生物活性的多肽片段和融合多肽。多肽并不限于特定的长度，例如，它们可包含全长蛋白多肽或全长蛋白多肽的片段，且可包含多肽的翻译后修饰，例如，糖基化、乙酰化、磷酸化等，以及其他本领域已知的天然存在的和非天然存在的修饰。可使用各种熟知的重组和 / 或合成技术中的任何一种制备本发明的多肽，其示例性实例进一步讨论如下。在一个实施方案中，所述 Wnt 多肽为截短的 Wnt1、Wnt2、Wnt2b/13、Wnt3、Wnt3a、Wnt4、Wnt5a、Wnt5b、Wnt6、Wnt7a、Wnt7b、Wnt8、Wnt8a、Wnt8b、Wnt9a、Wnt9b、Wnt10a、Wnt10b、Wnt11 或 Wnt16 多肽。

[0199] 在另一个实施方案中，所述 Wnt 多肽为 Fc- 融合多肽，其包含 Wnt1、Wnt2、Wnt2b/13、Wnt3、Wnt3a、Wnt4、Wnt5a、Wnt5b、Wnt6、Wnt7a、Wnt7b、Wnt8、Wnt8a、Wnt8b、Wnt9a、Wnt9b、Wnt10a、Wnt10b、Wnt11 或 Wnt16 多肽的全部或生物活性片段。

[0200] 在一个优选的实施方案中，所述 Wnt 多肽为 Wnt7a 多肽、截短的 Wnt7a 多肽或 Wnt7a Fc- 融合多肽，或它们的组合。

[0201] 如本文所用，术语“Wnt7a 多肽”是指 Wnt7a 蛋白，其具有对应于野生型 Wnt7a 序列的多肽序列。在一些实施方案中，术语“Wnt7a 多肽”是指 Wnt7a 多肽、截短的 Wnt7a 多肽、具有生物活性的 Wnt7a 多肽片段或 Wnt7a 融合多肽，其具有与参考 Wnt7a 序列有至少约 65%、66%、67%、68%、69%70%、71%、72%、73%、74%、75%、76%、77%、78%、79%、80%、81%、82%、83%、84%、85%、86%、87%、88%、89%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99% 或约 100% 的同一性的 Wnt7a 氨基酸序列。可针对至少约 10、25、50、75、100、125、150、175、200、300 个或更多个连续氨基酸进行同一性评估，或者可针对全长序列进行评估。确定 % 同一性或 % 同源性的方法为本领域已知，为此目的可使用任何合适的方法。Wnt7a 多肽的示例性实例如 SEQ ID NO:2-23 所示。

[0202] 然而，在具体的实施方案中，本发明的 Wnt 多肽经过改造，以便它们包含 N 端和 / 或 C 端缺失或截短，且在具体的实施方案中，所述 Wnt 多肽包含 N 端和 / 或 C 端缺失或截短，并保留了非经典的 Wnt 信号转导活性。在一些实施方案中，所述 Wnt 多肽包含 N 端和 / 或 C 端缺失或截短，缺少一个或多个脂化位点，并保留了非经典的 Wnt 信号转导活性。在具体的实施方案中，所述 Wnt 多肽为 Wnt7a 多肽，其包含 N 端和 / 或 C 端缺失或截短，并保留了非经典的 Wnt 信号转导活性。在一些实施方案中，所述 Wnt7a 多肽包含 N 端和 / 或 C 端缺失或截短，缺少一个或多个脂化位点，并保留了非经典的 Wnt 信号转导活性。

[0203] 如本文所用，术语“截短的 Wnt 多肽”或“包含 N 端和 / 或 C 端截短或缺失的 Wnt 多肽”可互换使用，并指缺少 N 端或 C 端氨基酸残基的 Wnt 多肽或 Wnt 多肽的生物活性片段

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或其变体，或者包含一个或多个氨基酸缺失的其同源物、间接同源物或直接同源物。在本发明的具体实施方案中，截短的 Wnt 多肽包含一个或多个氨基酸缺失，但产生了保留 Wnt 生物活性的多肽。在具体的实施方案中，截短的 Wnt 多肽保留了至少 100%、至少 90%、至少 80%、至少 70%、至少 60%、至少 50%、至少 40%、至少 30%、至少 20%、至少 10% 或至少 5% 的天然存在的 Wnt 多肽活性。

[0204] 如本文所用，术语“N 端缺失”和“N 端截短”通常可互换使用，并指从多肽中缺失了 N- 端氨基酸。例如 :包含 349 个氨基酸且具有 220 个氨基酸的 N- 端缺失的多肽所产生的多肽，其包含所述所肽的 129 个 C 端氨基酸 ;包含 349 个氨基酸且具有 234 个氨基酸的 N 端缺失的多肽所产生的多肽，其包含所述所肽的 115 个 C 端氨基酸 ;以及包含 349 个氨基酸且具有 263 个氨基酸的 N 端缺失的多肽所产生的多肽，其包含所述所肽的 86 个 C 端氨基酸。在具体的实施方案中，根据本发明的 Wnt 多肽，例如 Wnt7a，包含至少 220、221、222、223、224、225、226、227、228、229、230、231、232、233、234、235、236、237、238、239、240、241、242、243、244、245、246、247、248、249、250、251、252、253、254、255、256、257、258、259、260、261、262、263、264、265、266、267、268、269、270、271、272、273、274、275、276、277、278、279、280、281、282、283 或 284 个 N 端氨基酸的 N 端缺失或截短。在具体的实施方案中，包含 N 端截短的 Wnt 多肽还将包含一个或多个 C 端氨基酸截短或缺失。

[0205] 在具体的实施方案中，根据本发明的 Wnt 多肽，包含足以消除一个或多个 Wnt 脂化位点的 N 端缺失或截短。在某个实施方案中，Wnt 多肽包含至少 50、60、70、80、90、100、110、120、130、140、150、160、170、180、190、200、210、220、230、240、250、260、270、280、290 或 300 个 N 端氨基酸的 N 端缺失。

[0206] 如本文所用，术语“C 端缺失”和“C 端截短”通常可互换使用，且是指从多肽缺失一个或多个 C 端氨基酸。例如 :包含 349 个氨基酸且具有 10 个氨基酸的 C 端缺失的多肽所产生的多肽，其包含所述所肽的 339 个 N 端氨基酸 ;以及包含 349 个氨基酸且具有 20 个氨基酸的 C 端缺失的多肽所产生的多肽，其包含所述所肽的 329 个 N 端氨基酸。在具体的实施方案中，根据本发明的 Wnt 多肽包含至少 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49 或 50 个 C 端氨基酸的 C 端缺失或截短。在具体的实施方案中，包含 C 端截短的 Wnt 多肽还将包含一个或多个 N 端氨基酸截短或缺失。

[0207] 在具体的实施方案中，根据本发明的截短的 Wnt 多肽包含一个或多个 N 端氨基酸截短和一个或多个 C 端氨基酸截短，如本文其他地方所述。在某些实施方案中，截短的 Wnt 多肽包含约 10 个至约 300 个 N 端氨基酸的 N- 端缺失或截短和约 1 个至约 50 个 C 端氨基酸的 C 端缺失或截短。

[0208] 在某些实施方案中，截短的 Wnt 多肽包含约 220 个至约 284 个 N 端氨基酸的 N 端缺失或截短和约 1 个至约 50 个 C 端氨基酸的 C 端缺失或截短。

[0209] 在一个实施方案中，本发明包括这样的 Wnt 多肽，其包含含有一个或多个 N 端氨基酸截短和一个或多个 C 端氨基酸截短的 Wnt 多肽的最小生物活性片段，如本文其他地方所述。如本文所用，术语“最小活性片段”或“最小生物活性片段”是指 Wnt 多肽片段保留了至少 100%、至少 90%、至少 80%、至少 70%、至少 60%、至少 50%、至少 40%、至少 30%、至少 20%、至少 10% 或至少 5% 的天然存在的 Wnt 多肽活性。在具体的实施方案中，本发明包括最小生物

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活性 Wnt 片段，其包含 Wnt 多肽的 60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128 或 129 个连续氨基酸。

[0210] 在具体的实施方案中，所述天然存在的 Wnt 多肽活性或 Wnt 生物活性是非经典的 Wnt 信号转导活性。在具体的实施方案中，所述 Wnt7a 生物活性是非经典的 Wnt7a 信号转导活性。

[0211] 在另一个实施方案中，提供了最小生物活性 Wnt 片段，其包含 SEQ ID NO:3 所示氨基酸序列的 60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128 或 129 个连续氨基酸。

[0212] 在具体的实施方案中，Wnt 多肽的生物活性片段可为这样的多肽片段，其为本领域已知的、或本文引用或以其他方式公开的 Wnt 多肽氨基酸序列的例如，30、35、40、45、50、55、60、0、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128、129、130、131、132、133、134、135、136、137、138、139、140、141、142、143、144、145、146、147、148、149 或 150 个连续或非连续氨基酸，包括其间的所有整数（例如，101、102、103）和范围（例如，50-75，75-100，125-150）。在某些实施方案中，具有生物活性的 Wnt 多肽片段包含经典的活性相关序列、结构域或天然存在的 Wnt 多肽的基序。在某些实施方案中，根据本发明的 Wnt 多肽包含一个或多个 N 端氨基酸截短和一个或多个 C 端氨基酸截短，如本文其他地方所述。

[0213] 在具体的实施方案中，Wnt7a 多肽的生物活性片段可为这样的多肽片段，其为本文所述 Wnt7a 多肽中任一多肽所示氨基酸序列的例如，30、35、40、45、50、55、60、0、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128 或 129 个连续或非连续氨基酸，包括其间的所有整数（例如，101、102、103）和范围（例如，50-75、75-100、100-129）。在某些实施方案中，具有生物活性的 Wnt7a 多肽片段包含经典的活性相关序列、结构域或天然存在的 Wnt7a 多肽的基序。在某些实施方案中，根据本发明的 Wnt7a 多肽包含一个或多个 N 端氨基酸截短和一个或多个 C 端氨基酸截短，如本文其他地方所述。

[0214] 在某些实施方案中，截短的 Wnt 多肽包含约 220 个至约 284 个 N 端氨基酸的 N 端缺失或截短，包括其间的所有整数和范围（例如，221、222、223、224、225），以及约 1 个至约 50 个 C 端氨基酸的 C 端缺失或截短，包括其间的所有整数和范围（例如，1、2、3、4、5），只要所述截短的 Wnt7a 多肽保留了天然存在的 Wnt7a 多肽的活性。通常，所述生物活性片段具有不小于约 1%、5%、10%、20%、30、40%、50%、60%、70%、80%、90% 或 100% 的其所源自的天然存在 Wnt7a 多肽的活性，如非经典的 Wnt 信号转导活性。

[0215] 在一些实施方案中，截短的 Wnt 多肽，例如具有生物活性的 Wnt 多肽片段以至少约

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1%、5%、10%、20%、30、40%、50%、60%、70%、80%、90% 或 100% 的天然存在的 Wnt 多肽与相同细胞结合伴侣的结合亲和力，结合至一个或多个细胞结合伴侣，例如卷曲受体。在一些实施方案中，截短的 Wnt 多肽对于选定的细胞结合伴侣（尤其是参与经典活动的结合伴侣）的结合亲和力，可为天然存在的 Wnt 多肽的相应结合亲和力的至少约 1.5x、2x、2.5x、3x、3.5x、4x、4.5x、5x、6x、7x、8x、9x、10x、15x、20x、25x、30x、40x、50x、60x、70x、80x、90x、100x、200x、300x、400x、500x、600x、700x、800x、900x、1000x 或更多倍强（包括其间的所有整数）。

[0216] 本发明还包括 Wnt 多肽、截短的 Wnt 多肽、具有生物活性的 Wnt 多肽片段和 Wnt 融合多肽，其包含一个或多个氨基酸突变、添加或替换。在具体的实施方案中，本发明的 Wnt 多肽包含一个或多个氨基酸突变、添加和 / 或替换，但其保留或具有增加的 Wnt 生物活性。优选地，Wnt 多肽包含一个或多个氨基酸突变、添加和 / 或替换，且保留至少 100%、至少 90%、至少 80%、至少 70%、至少 60%、至少 50%、至少 40%、至少 30%、至少 20%、至少 10% 或至少 5% 的天然存在的 Wnt 活性。

[0217] 本发明还包括 Wnt7a 多肽、截短的 Wnt7a 多肽、具有生物活性的 Wnt7a 多肽片段和 Wnt7a 融合多肽，其包含一个或多个氨基酸突变、添加或替换。在具体的实施方案中，本发明的 Wnt7a 多肽包含一个或多个氨基酸突变、添加和 / 或替换，但其保留或具有增加的 Wnt7a 生物活性。优选地，Wnt7a 多肽包含一个或多个氨基酸突变、添加和 / 或替换，且保留至少 100%、至少 90%、至少 80%、至少 70%、至少 60%、至少 50%、至少 40%、至少 30%、至少 20%、至少 10% 或至少 5% 的天然存在的 Wnt7a 活性。

[0218] 如本文所用，术语“天然存在的”是指可在自然界中发现的多肽或多核苷酸序列。例如，天然存在的多肽或多核苷酸序列可为这样的多肽或多核苷酸序列，其在生物体中存在并可从生物体分离，且其没有在实验室中被人为特意修饰。术语“野生型”和“天然存在的”通常可互换使用。

[0219] 如本文所用，Wnt 多肽，例如保留了“天然存在的 Wnt 活性”、“天然存在的 Wnt7a 活性”、“正常 Wnt 活性”或“未修饰的 Wnt 活性”的 Wnt7a、其截短物、其生物活性片段以及 Wnt 融合多肽，是指修饰的 Wnt 多肽，例如 Wnt7a，其产生的生理响应或其具有的物理或药代动力学特性为相应天然存在的 Wnt 多肽例如 Wnt7a 的生理响应或物理或药代动力学特性的至少 100%、至少 90%、至少 80%、至少 70%、至少 60%、至少 50%、至少 40%、至少 30%、至少 20%、至少 10% 或至少 5%。在一些实施方案中，本发明的 Wnt7a 多肽保留了非经典的 Wnt 信号转导活性。

[0220] 在本发明的上下文中，截短的多肽、其生物活性片段或变体、或其同源物、其间接同源物、或其直接同源物、或融合多肽，当展示约 10%、20%、30%、40% 或 50% 的野生型蛋白活性，优选至少 50%、至少 55%、至少 60%、至少 65%、至少 70%、至少 75% 或至少 80% 的野生型蛋白活性时，其被认为具有与野生型蛋白至少基本上相同的活性。在具体的实施方案中，所述截短的多肽、其生物活性片段或变体、或其同源物、其间接同源物、或其直接同源物、或融合多肽，展示至少 70%、至少 80%、至少 90%、至少 95% 或约 100% 的野生型蛋白活性。在某些实施方案中，可获得大于野生型活性的活性。

[0221] 截短的 Wnt 多肽、具有生物活性的 Wnt 多肽片段或其变体、或其同源物、其间接同源物、或其直接同源物、或 Wnt 融合多肽的活性，例如可通过测量其模拟野生型 Wnt 生物活性的能力来确定，例如，通过刺激所述 Wnt 信号转导通路如通过促进对称的干细胞扩增或

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细胞生长，并将所述能力与野生型蛋白的活性进行比较。测量和定征干细胞分裂，例如卫星干细胞分裂，以及细胞生长，例如肌肉肥大的方法为本领域已知。

[0222] 本发明的截短的多肽可包括多肽变体。如本文所用，术语“变体”是指通过修饰、添加、缺失或替换至少一个氨基酸残基而区别于参考多肽的多肽，如本文其他地方所讨论，以及如本领域所理解。在某些实施方案中，多肽变体通过一个或多个氨基酸替换（例如，1、2、3、4、5 个或更多个替换）而区别于参考多肽，所述替换可为保守的或非保守的替换。例如，在各种实施方案中，可在发现于天然存在的 Wnt 多肽中的任何氨基酸残基中进行一个或多个保守或非保守替换。在其他具体实施方案中，Wnt 多肽变体包含一个或多个氨基酸添加、缺失或替换，以便增加 Wnt 通路信号转导活性，和 / 或相比天然存在的 Wnt 多肽，增加本发明 Wnt 多肽的稳定性、溶解度、全身性递送和 / 或组织吸收。

[0223] 为产生这类变体，例如根据表 1，本领域技术人员，例如可改变编码 DNA 序列的一个或多个密码子。

[0224] 表 1- 氨基酸密码子

[0225]

氨基酸	密码子			
丙氨酸	GCA	GCC	GCG	GCU
半胱氨酸	UGC	UGU		
天冬氨酸	GAC	GAU		
谷氨酸	GAA	GAG		
苯丙氨酸	UUC	UUU		
甘氨酸	GGA	GGC	GGG	GGU
组氨酸	CAC	CAU		
异亮氨酸	AUA	AUC	AUU	

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[0226]

氨基酸	密码子					
赖氨酸	AAA	AAG				
亮氨酸	UUA	UUG	CUA	CUC	CUG	CUU
甲硫氨酸	AUG					
天冬酰胺	AAC	AAU				
脯氨酸	CCA	CCC	CCG	CCU		
谷氨酰胺	CAA	CAG				
精氨酸	AGA	AGG	CGA	CGC	CGG	CGU
丝氨酸	AGC	AGU	UCA	UCC	UCG	UCU
苏氨酸	ACA	ACC	ACG	ACU		
缬氨酸	GUA	GUC	GUG	GUU		
色氨酸	UGG					
酪氨酸	UAC	UAU				

[0227] 确定哪个氨基酸残基可被替换、插入或缺失而不丧失生物学或免疫学活性的指导，可使用本领域熟知的计算机程序如 DNASTAR™ 软件来找到。如需要，可进行氨基酸替换以从多肽改变和 / 或去除功能基团。可选择地，本文公开的蛋白变体中的氨基酸改变可为保守氨基酸改变，即带类似电荷或不带电荷的氨基酸的替换。保守氨基酸改变涉及其侧链相关联的氨基酸家族成员之一的替换。天然存在的氨基酸通常分为 4 个家族 :酸性的（天冬氨酸盐、谷氨酸盐）、碱性的（赖氨酸、精氨酸、组氨酸）、非极性的（丙氨酸、缬氨酸、亮氨酸、异亮氨酸、脯氨酸、苯丙氨酸、甲硫氨酸、色氨酸），以及不带电荷的极性（甘氨酸、天冬酰胺、谷氨酰胺、半胱氨酸、丝氨酸、苏氨酸、酪氨酸）氨基酸。苯丙氨酸、色氨酸和酪氨酸有时被一起分类为芳香氨基酸。参见表 2。

[0228] 表 2- 保守氨基酸替换

[0229]

初始残基	保守替换
Ala(A)	Gly;Ser
Arg(R)	Lys

[0230]

初始残基	保守替换
Asn(N)	Gln;His

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Cys(C)	Ser
Gln(Q)	Asn
Glu(E)	Asp
Gly(G)	Ala;Pro
His(H)	Asn;Gln
Ile(I)	Leu;Val
Leu(L)	Ile;Val
Lys(K)	Arg;Gln;Glu
Met(M)	Leu;Tyr;Ile
Phe(F)	Met;Leu;Tyr
Ser(S)	Thr
Thr(T)	Ser
Trp(W)	Tyr
Tyr(Y)	Trp;Phe
Val(V)	Ile;Leu

[0231] 其他替换也是允许的，且可根据经验或按照其他已知的保守（或非保守）替换来确定。

[0232] 在作出这类改变时，可考虑到氨基酸的亲水指数。本领域通常了解亲水氨基酸指数在赋予蛋白相互作用的生物功能方面的重要性（Kyte 和 Doolittle，1982，通过引用并入本文）。本领域已知，某些氨基酸可被其他具有类似亲水指数或分数的氨基酸替换，且仍然产生具有类似生物活性的蛋白，即仍获得生物功能等效的蛋白。在作出这类改变时，优选替换那些亲水指数在 ±2 以内的氨基酸，尤其优选 ±1 以内的那些，甚至更尤其优选 ±0.5 以内的那些。本领域也了解，可基于亲水性有效地进行类似氨基酸的替换。

[0233] 本发明的多肽变体包括糖基化形式、与其他分子的集合性缀合物，以及与非相关化学基团（例如，聚乙二醇化的分子）的共价缀合物。如本领域所知，共价变体可通过将官能基（functionalities）与在所述氨基酸链或者 N 端或 C 端残基处发现的基团连接来制备。变体还包括等位变体、种变体和突变蛋白。在某些实施方案中，不影响蛋白功能活性的区域的截短或缺失也是变体。

[0234] 可通过本领域已知的方法来确定本发明的多肽中对于发挥功能很关键的氨基酸，如定点诱变或丙氨酸扫描诱变（Cunningham and Wells，Science244:1081-108

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5,1989)。对于配体-受体结合至关重要的位点也可通过结构分析来确定,如结晶化、核磁共振或光亲和标记(Smith et al.,J.Mol.Biol.224:899-904,1992 和 de Vos et al.Science255:306-312,1992)。

[0235] 某些改变不会显著影响蛋白的折叠或活性。本领域技术人员进行氨基酸替换的数目取决于许多因素,包括以上所述的那些。一般来说,对任何给定多肽的替换数目不会超过50、40、30、25、20、15、10、5 或 3 个。

[0236] 此外,多肽和/或突变蛋白的聚乙二醇化有望提供改良的特性,如增加的半衰期、溶解度和蛋白酶抗性。聚乙二醇化为本领域所熟知。

[0237] 序列同一性可用于比较两个多核苷酸或多肽序列的一级结构、描述第一序列相对于第二序列的一级结构,和/或描述序列关系如变体和同源物。当比对最大相关性时,序列同一性测量两个序列中相同的残基。比较多肽序列时,当比对最大一致性时,如果两个序列中的氨基酸序列相同,则两个序列被称为"相同的",如下所述。可使用计算机实现的算法来分析序列关系。两个或更多个多核苷酸或者两个或更多个多肽之间的序列关系可通过计算这些序列的最佳对齐方式并对比对中的匹配和空位打分来确定,其产生百分比序列同一性和百分比序列相似性。多核苷酸关系也可基于其每个编码的多肽的比较来描述。许多用于序列比较和分析的程序和算法是已知的。

[0238] 通常,通过在比较窗上比较序列来进行两个序列间的比较,以确定和比较局部区域的序列相似性。如本文所用,"比较窗"是指至少约 20 个连续位置,通常为 30 至约 75 个、40 至约 50 个位置的片段,其中两个序列进行最优比对后,可将序列与具有相同数目的连续位置的参考序列比较。

[0239] 当使用确定的氨基酸替换矩阵(例如,BLOSUM62)、空位存在罚分和空位延伸罚分进行相似性打分比对时,两个序列被"最优比对",以便达到这对序列可能的最高分数。氨基酸替换矩阵及其在定量两个序列之间的相似性中的应用为本领域熟知且描述于,例如 Dayhoff et al.(1978)A model of evolutionary change in proteins."Atlas of Proein Sequence and Structure,Vol.5,Suppl.3(ed.M.O.Dayhoff),pp.345-352.Natl.Biomed.Res.Found.,Washington,D.C. 和 Henikoff et al.(1992)Proc.Natl.Acad.Sci.USA89:10915-10919。BLOSUM62 矩阵(图 10)在序列比对方案如 Gapped BLAST2.0 中通常被用作默认的打分替换矩阵。空位存在罚分应用于在一个比对的序列中引入单个氨基酸空位,而空位延伸罚分应用于插入至已开放的空位中的每个额外的空氨基酸位置。比对界定为比对开始和结束的每个序列的氨基酸位置,并且任选地通过在一个或两个序列中插入一个空位或多个空位,以便达到可能的最高分数。尽管最佳比对和打分可以手动完成,通过使用计算机实现的比对算法可使该过程更为便利,例如 Gapped BLAST2.0,其描述于 Altschul et al.,(1997)Nucleic Acids Res.25:3389-3402,且在美国国家生物技术信息中心网站(www.ncbi.nlm.nih.gov)向公众提供。可使用例如 PSI-BLAST 来进行最佳比对,包括多重比对,其通过 www.ncbi.nlm.nih.gov 提供,且描述于 Altschul et al.,(1997)Nucleic Acids Res.25:3389-3402。

[0240] 此外,比较序列的最佳比对可通过下述方法进行:Smith and Waterman(1981)Add.APL.Math2:482 的局部标识算法、Needleman and Wunsch(1970)J.Mol.Biol.48:443 的同一性比对算法、Pearson and Lipman(1988)Proc.Nat'l Acad.Sci.USA85:2444 的

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相似性查找法、这些算法的计算机实现（威斯康星遗传学软件包 (Wisconsin Genetics Software Package) 中 的 GAP、BESTFIT、BLAST、FASTA 和 TFASTA，Genetics Computer Group(GCG),575Science Dr.,Madison,WI)，或通过目测。在一个实施方案中，可使用 BLAST 比对工具来评估多核苷酸和 / 或多肽。局部比对仅由一对序列片段组成，其分别来自每个待比对的序列。Smith-Waterman 或 Sellers 算法的修改可发现所有通过延伸或修剪无法提高分数的片段对，称为高分片段对 (HSPs)。BLAST 比对的结果包括统计学测量，以显示仅从偶然可预期到 BLAST 分数的可能性。

[0241] 原始分数 S，计算自与每个比对的序列相关的空位和替换的数目，其中更高的相似性分数表明为更显著的对齐。替换分数可通过查表来给出（参见 PAM,BLOSUM)。

[0242] 空位分数通常计算为空位开放罚分 G 和空位延伸罚分 L 的总和。对于长度为 n 的空位，空位罚分 (gap cost) 为 G+Ln。根据经验选择空位罚分 G 和 L，但通常选择高数值的 G(10-15)，例如 11，以及低数值的 L(1-2)，例如 1。

[0243] 比特 (bit) 分数 S' 源自原始比对分数 S，其中考虑了所用打分系统的统计特征。针对打分系统将比特分数标准化，从而，其可用于比较来自不同查找的比对分数。术语“比特分数”和“相似性分数”可互换使用。比特分数显示该比对有多好；分数越高，对齐越好。

[0244] E- 值或预期值，描述了具有类似分数的序列通过偶然性在数据库中出现的可能性。其为预期通过偶然性在数据库搜索中出现的具有等于或好于 S 分数的不同比对的数目的预测。E- 值越小，该比对越显著。例如，具有 e^{-117} 的 E 值的比对意味着具有类似分数的序列仅凭偶然性非常不可能出现。此外，比对随机对的氨基酸的预期分数需要为负值，否则长的比对将倾向于具有高分数，不论比对的片段是否相关。此外，BLAST 算法使用合适的替换矩阵、核苷酸或氨基酸，且对于有空位的比对，使用空位生成和延伸罚分。例如，多肽序列的 BLAST 比对和比较通常使用 BLOSUM62 矩阵进行，所用的空位存在罚分为 11，而空位延伸罚分为 1。

[0245] 在一个实施方案中，从使用 BLOSUM62 矩阵进行的 BLAST 分析报告序列相似性分数，所用的空位存在罚分为 11，而空位延伸罚分为 1。

[0246] 在一个具体实施方案中，本文提供的序列同一性 / 相似性分数是指使用 GAP 第 10 版 (GCG,Accelrys,San Diego,Calif.) 采用以下参数获得的值：采用 50 的空位权重和 3 的长度权重以及 nwsgapdna.cmp 打分矩阵的核苷酸序列 % 同一性和 % 相似性；采用 8 的空位权重和 2 的长度权重以及 BLOSUM62 打分矩阵的氨基酸序列 % 同一性和 % 相似性 (Henikoff and Henikoff(1992)Proc Natl Acad Sci USA89:10915-10919)。GAP 使用 Needleman and Wunsch(1970)J Mol Biol48:443-453 的算法查找两个完整序列的比对，其使匹配数目最大化，并使空位数目最小化。

[0247] 在一个具体实施方案中，所述截短的 Wnt 多肽包含可与 SEQ ID NO:3-5 中任一序列的多肽序列进行最优比对的氨基酸序列，所产生的 BLAST 比特分数或序列相似性分数为至少 170、171、172、173、174、175、176、177、178、179、180、181、182、183、184、185、186、187、188、189、190、191、192、193、194、195、196、197、198、199、200、201、202、203、204、205、206、207、208、209、210、211、212、213、214、215、216、217、218、219、220、221、222、223、224、225、226、227、228、229、230、231、232、233、234、235、236、237、238、239、240、241、242、243、244、245、256、247、248、249、250、251、252、253、254、255、256、257、258、259、260、261、262、

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263、264、265、266、267、268、269、270、271、272、273、274 或 275，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0248] 在一个具体实施方案中，所述截短的 Wnt 多肽包含可与 SEQ ID NO:3-5 中任一序列的多肽序列进行最优比对的氨基酸序列，所产生的 BLASTe- 值分数为至少 e-52、e-53、e-54、e-55、e-56、e-57、e-58、e-59、e-60、e-61、e-62、e-63、e-64、e-65、e-66、e-67、e-68、e-69、e-70、e-71、e-72、e-73、e-74、e-75、e-76、e-77、e-78、e-79 或 e-80，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0249] 在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的相似性分数为 220-275，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的 e- 值分数为 e-74 至 2e-78，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0250] 在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:4 的多肽序列进行最优比对的氨基酸序列，所产生的相似性分数为 210-242，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的 e- 值分数为 e-66 至 e-69，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0251] 在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:5 的多肽序列进行最优比对的氨基酸序列，所产生的相似性分数为 171-184，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的 e- 值分数为 e-52 至 3e-52，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0252] 在某些实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的相似性分数为至少 220，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的 e- 值分数为至少 e-74，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0253] 在某些实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:4 的多肽序列进行最优比对的氨基酸序列，所产生的相似性分数为至少 210，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的 e- 值分数为至少 e-66，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0254] 在某些实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:5 的多肽序列进行最优比对的氨基酸序列，所产生的相似性分数为至少 171，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。在具体的实施方案中，截短的 Wnt 多肽包含可与 SEQ ID NO:3 的多肽序列进行最优比对的氨基酸序列，所产生的 e- 值分数为至少 e-52，其中所述 BLAST 比对使用 BLOSUM62 矩阵，空位存在罚分为 11，而空位延伸罚分为 1。

[0255] 在另一个示例性方法中，“序列同一性百分比”通过在至少 20 个位置的比较窗上比较两个最优比对的序列来确定，其中相比用于两个序列的最佳比对的参考序列（其不

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包含添加或缺失），在所述比较窗中的多肽序列部分可包含 20% 或更少的，通常 5%-15% 或 10%-12% 的添加或缺失（即，空位）。通过以下方式计算所述百分比：确定两个序列中出现相同氨基酸残基的位置数目以产生匹配位置数，用匹配位置数除以参考序列的总位置数（即，窗的大小），并将该结果乘以 100 得到序列同一性百分比。

[0256] E. 融合多肽

[0257] 在各种实施方案中，本发明部分地包括，融合多肽和编码融合多肽的多核苷酸。在一个实施方案中，所述融合多肽包含截短的 Wnt 多肽、具有生物活性的 Wnt 多肽片段和 / 或这样的肽，其还包含一个或多个氨基酸突变、替换和 / 或添加，如本文其他地方所述。在一个优选的实施方案中，所述 Wnt 多肽为 Wnt7a。在一个具体实施方案中，所述 Wnt 多肽为 Wnt7a 融合多肽，其包含 N 端和 / 或 C 端缺失或截短并保留了非经典的 Wnt 信号转导活性。在一些实施方案中，所述 Wnt7a 多肽为 Wnt7a 融合多肽，其包含 N 端和 / 或 C 端缺失或截短，缺少一个或多个脂化位点并保留了非经典的 Wnt 信号转导活性。在优选的实施方案中，所述融合多肽保留了非经典的 Wnt 信号转导活性。

[0258] 在具体的实施方案中，本发明的 Wnt 融合多肽促进干细胞扩增，并促进细胞和 / 或组织形成、再生、维持和修复。本发明的 Wnt 融合多肽用于在人的受损和患病的肌肉组织中促进修复和再生的方法中。

[0259] 融合多肽可包含在所述蛋白 N 末端的信号肽，其在翻译同时或翻译后指导所述蛋白、截短的 Wnt 多肽或具有生物活性的 Wnt 多肽片段的转移。融合多肽还可包含连接子或间隔子、Fc 结构域、一个或多个蛋白酶切割位点，或一个或多个表位标签或其他序列，以便于所述多肽的合成、纯化或产生。

[0260] 融合多肽和融合蛋白是指本发明多肽被直接或通过氨基酸连接子共价连接至一个或多个异源多肽序列（融合伴侣）。形成所述融合蛋白的多肽通常以 C 端连接至 N 端，尽管它们也可以 C 端连接至 C 端、N 端连接至 N 端，或 N 端连接至 C 端。所述融合蛋白的多肽可为任何顺序。

[0261] 为基本上任何所需目的，可设计和包含所述融合伴侣，只要它们不给所述多肽的所需活性带来不利影响。例如，在一个实施方案中，可选择融合伴侣以便增加所述蛋白的溶解度，从而促进 Wnt 多肽的生产和 / 或纯化，和 / 或促进 Wnt 的全身性递送和 / 或组织吸收。融合多肽可通过化学合成方法制备，或通过两个基团之间的化学连接制备，或者通常可采用其他标准技术制备。在一个实施方案中，Wnt 例如 Wnt7a 融合多肽，包含信号肽和截短的 Wnt 多肽或具有生物活性的 Wnt 多肽片段。

[0262] 在一个具体实施方案中，Wnt 例如，Wnt7a 融合多肽包含信号肽、如本文其他地方所述的截短的 Wnt 多肽或具有生物活性的 Wnt 多肽片段、蛋白酶切割位点和表位标签。

[0263] 如本文所用，术语“信号肽”是指确保进入分泌途径的先导序列。对于工业化生产分泌蛋白，所述待生产的蛋白需要从宿主细胞或宿主生物体中有效地分泌出来。所述信号肽可为例如，所述待生产蛋白的天然信号肽、异源信号肽，或天然和异源信号肽的杂合体。许多信号肽被用于生产分泌蛋白。

[0264] 用于本发明融合多肽的信号肽的示例性实例，包括但不限于：CD33 信号肽；免疫球蛋白信号肽，例如 IgGκ 信号肽或 IgGμ 信号肽；生长激素信号肽；红细胞生成素信号肽；白蛋白信号肽；分泌型碱性磷酸酶信号肽和病毒信号肽，例如轮状病毒 VP7 糖蛋白信号

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肽。

[0265] 在具体的实施方案中，本发明的融合多肽包含蛋白酶切割位点和表位标签，以促进截短的 Wnt 多肽，例如 Wnt7a 的纯化和生产。蛋白酶切割位点的位置通常位于所述 Wnt 多肽 C 端和表位标签之间，以便于在将所述 Wnt 递送至细胞或组织前去除异源序列。

[0266] 可用于本发明融合蛋白的异源蛋白酶切割位点的示例性实例，包括但不限于：烟草蚀纹病毒 (TEV) 蛋白酶切割位点、肝素切割位点、凝血酶切割位点、肠激酶切割位点和 Xa 因子切割位点。

[0267] 可用于本发明融合蛋白的表位标签的示例性实例，包括但不限于：HIS6 表位、MYC 表位、FLAG 表位、V5 表位、VSV-G 表位和 HA 表位。

[0268] 融合蛋白通常可采用标准技术制备。例如，编码所需融合物的多肽元件的 DNA 序列可分别组装，并连接至合适的表达载体中。编码一个多肽元件的 DNA 序列的 3’ 端通过或不通过肽连接子连接至编码第二多肽元件的 DNA 序列的 5’ 端，以便所述序列的阅读框同相 (in phase)。这允许翻译为保留了两个多肽元件生物活性的单融合蛋白。

[0269] 如需要，也可使用肽连接子序列以足够距离分隔所述融合多肽元件，以确保每个多肽折叠为其二级和三级结构。通过使用本领域熟知的标准技术，这样的肽连接子序列被整合至所述融合蛋白。可基于以下因素选择某些肽连接子序列：(1) 其采用柔性伸展构象的能力；(2) 其采用能够与第一和第二多肽上的功能表位相互作用的二级结构的能力；以及 (3) 缺少可与多肽功能表位反应的疏水或带电荷残基。优选的肽连接子序列含有 Gly、Asn 和 Ser 残基。其他接近中性的氨基酸，如 Thr 和 Ala 也可用于连接子序列。可有效用作连接子的氨基酸序列包括公开于以下文献中的序列：Maratea et al., Gene40:3946(1985)；Murphy et al., Proc. Natl. Acad. Sci. USA83:82588262(1986)；美国专利第 4,935,233 号和美国专利第 4,751,180 号。所述连接子序列通常可为 1 至约 50 个氨基酸长度。当所述第一和第二多肽具有可用于分隔所述功能结构域和防止空间位阻的非必需 N 端氨基酸区域时，不需要连接子序列。这两个编码序列可直接融合，而无需任何连接子或通过使用由五联体 Gly-Gly-Gly-Gly-Ser 重复 1-3 次组成的柔性多聚连接子。这样的连接子通过插入至 VH 和 VL 之间已被用于构建单链抗体 (scFv)(Bird et al., 1988, Science242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. U. S. A. 85:5979-5883)。所述连接子被设计为使得形成单链抗体的可变区的两个 β - 折叠之间能够正确地相互作用。可使用的其他连接子包括 Glu-Gly-Lys-Ser-Ser-Gly-Ser-Gly-Ser-Glu-Ser-Lys-Val-Asp(Chaudhary et al., 1990, Proc. Natl. Acad. Sci. U. S. A. 87:1066-1070) 和 Lys-Glu-Ser-Gly-Ser-Val-Ser-Ser-Glu-Gln-Leu-Ala-Gln-Phe-Arg-Ser-Leu-A sp(Bird et al., 1988, Science242:423-426)。

[0270] 通常，多肽、融合多肽（及其编码多核苷酸）和细胞是分离的。“分离的”多肽或多核苷酸为从其原始环境中去除的多肽或多核苷酸。例如，如本文所用，“分离的肽”或“分离的多肽”等是指在体外将肽或多肽分子从细胞环境和从与所述细胞其他组分的结合中分离和 / 或纯化出来，即其不大量地与体内物质结合。类似地，如本文所用，“分离的多核苷酸”是指已经被纯化而与在天然存在状态下和它侧接的序列分离，例如，从正常情况下与该片段邻接的序列上脱离的 DNA 片段。如果，例如多核苷酸被克隆至不是其自然环境的部分的载体中，则多核苷酸被认为是分离的。“分离的细胞”是指获自体内组织或器官且基本上无

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胞外基质的细胞。优选地，如果其至少约 60% 纯、至少约 70% 纯、至少约 80% 纯、至少约 90% 纯，更优选地至少约 95% 纯，以及最优选地至少约 99% 纯，则多肽、多核苷酸或细胞是分离的。

[0271] 在具体的实施方案中，多肽可在细胞中或合成性表达为融合蛋白，然后纯化。

[0272] 在其他实施方案中，一个或多个多肽可在它们于细胞中或合成性制备以后进行融合。通常，根据本领域已知的和本文描述的技术，任选地通过多种生物相容的聚合物或不相关的化学基团，可将在制备出融合物中的单个多肽后再融合的多肽共价地连接或缀合。如本领域所知，可通过将官能基 (functionalities) 与在氨基酸链或 N 端或 C 端残基中发现的基团连接来制备共价变体。此外，非肽聚合物（例如，至少 2 个共价连接的非肽部分）包括，例如，聚乙二醇 (PEG)、聚丙二醇、乙二醇和丙二醇的共聚物、聚氧乙烯多元醇、聚乙烯醇、寡糖、葡聚糖、聚乙烯乙醚、可生物降解聚合物、脂类聚合物、几丁质、透明质酸及它们的组合物，可作为间隔子或连接子来融合两个或更多个多肽序列。合适的非肽聚合物的实例包括但不限于，PEG、N-(2- 羟丙基）甲丙烯酰胺 (HPMA)、聚乙烯吡咯烷酮 (PVP) 和聚乙烯亚胺 (PEI)。

[0273] 如本文所用，术语“获自”是指样品例如，多核苷酸或多肽分离自或源自特定来源如重组宿主细胞。在另一个实施方案中，术语“获自”是指细胞分离自或源自诸如体内组织或器官的来源。

[0274] 在各种实施方案中，提供了包含截短的 Wnt 蛋白和 Fc 结构域的融合多肽。所述 Fc 结构域可融合至另一多肽例如，连接子多肽或 Wnt 多肽的 N 端或 C 端。所述 Fc- 融合物可作为分子伴侣 :提高蛋白稳定性和溶解度。所述 Fc- 融合物也可对体内药代动力学特性具有主要影响 :通过增加分子量、防止通过肾脏过滤排出以及通过存在于身体的许多细胞上的新生 Fc 受体来循环该蛋白，延长了该蛋白的半衰期。治疗性抗体和治疗性 Fc- 融合蛋白可通过刺激或抑制免疫反应来起作用 :与效应细胞上 Fcγ 受体相互作用产生免疫功能如抗体依赖性细胞介导的细胞毒性 (ADCC) 或补体依赖性细胞毒性 (CDC)。

[0275] 4 种人 IgG 同型物 (1、2、3 和 4) 以不同的亲和力结合活化性 Fcγ 受体 (FcγRI、FcγRIIa、FcγRIIIa)、抑制性 FcγRIIb 受体和补体 (C1q) 的第一元件，产生极不相同的效应功能。例如，IgG1 诱导有效的 ADCC 和 CDC 反应，而 IgG2 和 IgG4 同种型对于正调控性 Fcγ 受体具有明显降低的亲和力。因此，当需要特定效应功能时，可使用特定 IgG 同种型的 Fc 结构域。当一种免疫反应非治疗上所需时，可使用 IgG4 亚型 Fc 或经改造而具有降低的效应功能的其他亚型 Fc 结构域。产生免疫反应的具体 CH2 和 CH3 结构域点突变为本领域所知 (Chames2009)。

[0276] 在具体的实施方案中，融合多肽包含截短的 Wnt 多肽和 Fc 结构域。在某些实施方案中，所述 Wnt-Fc 结构域融合多肽保留了 Wnt 生物活性，但并不诱导 ADCC 和 CDC 反应。所述 Fc 结构域可获自免疫球蛋白的任何类别——IgG、IgA、IgM、IgD 和 IgE。在一些实施方案中，Fc 区为野生型 Fc 区。在一些实施方案中，Fc 区为突变的 Fc 区。在一些实施方案中，Fc 区在 N 末端截短了 1、2、3、4、5、6、7、8、9 或 10 个氨基酸（例如，在铰链结构域）。

[0277] 在具体的实施方案中，本发明的 Wnt 融合多肽包含如本文其他地方所述的 N 端和 / 或 C 端截短的 Wnt 多肽或具有生物活性的 Wnt 多肽片段以及 Fc 结构域。在某些实施方案中，本发明的 Wnt 融合多肽包含如本文其他地方所述的信号肽、N 端和 / 或 C 端截短的 Wnt

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多肽或具有生物活性的 Wnt 多肽片段、蛋白酶和 Fc 结构域。在一些实施方案中，这些 Wnt 融合多肽包含 N 端和 / 或 C 端缺失或截短，缺少一个或多个脂化位点，并保留了非经典的 Wnt 信号转导活性。在优选的实施方案中，这些 Wnt-Fc 结构域融合蛋白不具有可检测的 ADCC 或 CDC 活性。在各种相关的实施方案中，相比天然的 Wnt 多肽，这些 Wnt-Fc 结构域融合蛋白保留了 Wnt 生物活性，并具有改良的生产、分泌和 / 或稳定性。

[0278] 在其他实施方案中，本发明的 Wnt7a 融合多肽包含如本文其他地方所述的 N 端和 / 或 C 端截短的 Wnt7a 多肽或具有生物活性的 Wnt7a 多肽片段和 Fc 结构域。在某些实施方案中，本发明的 Wnt7a 融合多肽包含如本文其他地方所述的信号肽、N 端和 / 或 C 端截短的 Wnt7a 多肽或具有生物活性的 Wnt7a 多肽片段、蛋白酶和 Fc 结构域。在一些实施方案中，这些 Wnt7a 融合多肽包含 N 端和 / 或 C 端缺失或截短，缺少一个或多个脂化位点并保留了非经典的 Wnt 信号转导活性。在优选的实施方案中，这些 Wnt7a-Fc 结构域融合蛋白不具有可检测的 ADCC 或 CDC 活性。在各种相关的实施方案中，相比天然的 Wnt7a 多肽，这些 Wnt7a-Fc 结构域融合蛋白保留了 Wnt 生物活性并具有改良的生产、分泌和 / 或稳定性。

[0279] F. 多核苷酸

[0280] 本发明还提供了编码本发明 Wnt 多肽的分离的多核苷酸。在各种实施方案中，本发明部分地包括，编码保留了 Wnt 生物活性的多肽截断物或具有生物活性的片段或 Wnt 融合多肽的 Wnt 多核苷酸，并且在一些实施方案中，其具有增加的 Wnt 信号转导活性。在具体的实施方案中，本发明的 Wnt 多核苷酸编码 Wnt 多肽，其促进干细胞扩增且促进细胞和 / 或组织形成、再生、维持和修复。

[0281] 本发明的 Wnt 多核苷酸适于临床规模上生产 Wnt 多肽并用于在人的受损和患病肌肉组织中促进修复和再生的方法。在某些实施方案中，Wnt 多核苷酸编码 Wnt 多肽，其缺少一个或多个用于所述 Wnt 多肽脂化的天然氨基酸。在某些具体的实施方案中，Wnt 多核苷酸编码截短的 Wnt 多肽，其包含一个或多个氨基酸缺失或 Wnt 多肽或 Wnt 融合多肽的 N 端和 / 或 C 端截短。在优选的实施方案中，所述 Wnt 多核苷酸编码 Wnt 融合多肽或 Wnt7a 多肽，其包含一个或多个氨基酸缺失或 N 端和 / 或 C 端截短，但保留了或具有增加的 Wnt 生物活性，如经典的和非经典的 Wnt 信号转导活性。

[0282] 可使用本领域已知的方案合成核酸，其描述于 Caruthers et al., 1992, Methods in Enzymology211, 3-19; Thompson et al., PCT 国际公开第 WO99/54459 号 ; Wincott et al., 1995, Nucleic Acids Res. 23, 2677-2684; Wincott et al., 1997, Methods Mol. Bio., 74, 59-68; Brennan et al., 1998, Biotechnol Bioeng., 61, 33-45; 和 Brennan, 美国专利第 6,001,311 号。

[0283] 通过“核苷酸”是指以 N- 糖苷键与磷酸化糖连接的杂环含氮碱基。本领域认为核苷酸包括天然（标准的）碱基和本领域熟知的修饰的碱基。这类碱基通常位于核苷酸糖基部分的 1’ 位置。核苷酸通常包括碱基、糖和磷酸基。核苷酸可在糖、磷酸和 / 或碱基部分处未经修饰或经修饰，也可互换地称为核苷酸类似物、修饰的核苷酸、非天然核苷酸、非标准核苷酸等（参见例如，Usman and McSwiggen, 同上 ; Eckstein et al., PCT 国际公开第 WO92/07065 号 ; Usman et al., PCT 国际公开第 WO93/15187 号 ; Uhlman&Peyman, 同上）。有几个本领域已知的修饰的核酸碱基实例，其概述于 Limbach et al., (1994, Nucleic Acids Res. 22, 2183-2196)。

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[0284] 如本文所用，术语“DNA”和“多核苷酸”和“核酸”是指已从特定物种的总基因组DNA分离的DNA分子。因此，编码多肽的DNA片段是指，DNA片段包含一个或多个编码序列，但其已基本上从获得该DNA片段的物种的总基因组DNA分离或纯化出来。术语“DNA片段”和“多核苷酸”包括DNA片段和此类片段的更小的片段以及重组载体，包括例如，质粒、粘粒、噬菌粒、噬菌体、病毒等。

[0285] 本领域技术人员应理解，本发明的多核苷酸序列可包括基因组序列、基因组外的和质粒编码的序列以及更小的改造的基因片段，其表达或可适用于表达蛋白、多肽、肽等。这类片段可为天然分离的、重组的或经人工合成改造的。

[0286] 本领域技术人员应理解，多核苷酸可为单链的（编码链或反义链）或双链的，且可为DNA（基因组的、cDNA或合成的）或RNA分子。其他编码性或非编码性序列可以但不必须存在于本发明的多核苷酸中，且多核苷酸可以但不必须连接至其他分子和/或支持材料。

[0287] 多核苷酸可包括天然序列（即，编码本发明的多肽或其部分的内源序列），或者可包括该序列的变体或生物学功能等同物。多核苷酸变体可包含一个或多个如本文其他地方所述的替换、添加、缺失和/或插入，优选地，以便变体编码缺少经典的脂化位点，但保留了，且在一些实施方案中具有增加的生物活性如通路信号转导活性的多肽。

[0288] 还包括与编码本发明多肽的多核苷酸杂交的多核苷酸。在“严格条件下”杂交描述了这样的杂交方案，其中彼此至少具有60%同一性的核苷酸序列保持杂交状态。高度严格的杂交条件是使得探针、引物或寡核苷酸仅能与其靶序列杂交的条件。严格条件是序列依赖性的且各不相同。适度严格条件是这样的条件，其使用比高度严格条件具有较少严格性的洗涤液和杂交条件(Sambrook,1989)，以便多核苷酸将与本发明核酸的整体、片段、衍生物或类似物杂交。适度严格条件描述于(Ausubel et al.,1987;Kriegler,1990)。低度严格条件是这样的条件，其使用比适度严格条件具有较少严格性的洗涤液和杂交条件(Sambrook,1989)，以便多核苷酸与本发明核酸的整体、片段、衍生物或类似物杂交。低度严格条件，如种间杂交的条件，描述于(Ausubel et al.,1987;Kriegler,1990;Shilo and Weinberg,1981)。

[0289] 在其他实施方案中，本发明提供分离的多核苷酸，其包含序列的各种长度的连续延伸，所述序列与编码本文所述多肽或融合多肽的多核苷酸相同或互补。例如，本发明提供的多核苷酸编码本发明多肽的至少约60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、86、87、88、89、90、91、92、93、94、95、96、97、98、99、100、101、102、103、104、105、106、107、108、109、110、111、112、113、114、115、116、117、118、119、120、121、122、123、124、125、126、127、128或129个连续氨基酸残基。本领域技术人员应理解，由于遗传密码的简并性，有许多核苷酸序列编码如本文所述的多肽，包括经最优化用于人和/或灵长类密码子选择的多核苷酸。此外，也可使用包含本文提供的多核苷酸序列的基因的等位基因。

[0290] 可使用多种成熟的已有技术中的任何一种鉴定、制备和/或操作本发明的多核苷酸组合物（通常参见，Sambrook et al.,Molecular Cloning:A Laboratory Manual,Cold Spring Harbor Laboratories,Cold Spring Harbor,NY,1989，以及其他类似文献）。

[0291] 多种表达载体/宿主系统是已知的，并可用于容纳和表达多核苷酸序列。这些包括但不限于，微生物如用重组噬菌体、质粒或粘粒DNA表达载体转化的细菌；用酵母表达载

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体转化的酵母；用病毒表达载体（例如，杆状病毒）感染的昆虫细胞系统；用病毒表达载体（例如，花椰菜花叶病毒 CaMV；烟草花叶病毒 TMV）或用细菌表达载体（例如，Ti 或 pBR322 质粒）转化的植物细胞系统；或哺乳动物细胞系统。

[0292] 存在于表达载体的“控制元件”或“调控序列”是该载体的非翻译区的那些—增强子、启动子、5’ 和 3’ 非翻译区—其与宿主细胞蛋白相互作用而进行转录和翻译。所述载体元件通常包括但不限于，下述中的一个或多个：信号序列、复制起始点、一个或多个标记基因、增强子元件、由宿主生物体识别的启动子和转录终止序列。也可使用特定启动信号来实现编码目标多肽的序列的更有效翻译。

[0293] 可针对其以期望的方式调控插入序列的表达或加工表达的蛋白的能力来挑选宿主细胞株。这类多肽修饰包括但不限于，乙酰化、羧化、糖基化、磷酸化、脂化和酰化。也可使用切割“前原 (prepro)”形式的蛋白的翻译后加工，从而促进正确的插入、折叠和 / 或功能。示例性哺乳动物宿主细胞如 CHO 细胞、COS 细胞、CV1 细胞、小鼠 L 细胞、小鼠 LSL 细胞、HeLa 细胞、MDCK 细胞、HT1080 细胞、BHK-21 细胞、HEK293 细胞、NIH-3T3 细胞、LM 细胞、YI 细胞、NSO 和 SP2/0 小鼠杂交瘤细胞等、Namalwa 细胞、RPMI-8226 细胞、Vero 细胞、WI-38 细胞、MRC-5 细胞或其他永生化和 / 或转化细胞，其具有针对这类翻译后活性的特定细胞结构和特征机制，可被挑选以确保外来蛋白的高表达以及正确修饰和加工。

[0294] 采用对产物有特异性的多克隆或单克隆抗体，检测和测量多核苷酸编码产物的表达的多种方案为本领域所知。实例包括酶联免疫吸附检测 (ELISA)、放射免疫检测 (RIA) 和荧光激活细胞分选术 (FACS)。这些和其他检测法还描述于 Hampton et al., Serological Methods, a Laboratory Manual(1990)， 以 及 Maddox et al., J. Exp. Med. 158:1211-1216(1983) 等。

[0295] 用目标多核苷酸序列转化的宿主细胞，可在适于表达和从细胞培养物回收所述蛋白的条件下培养。根据所用的序列和 / 或载体，由重组细胞产生的蛋白可被分泌或容纳于细胞内。

[0296] 除了重组生产方法，本发明的多肽及其片段可通过使用固相技术直接进行肽合成来制备 (Merrifield, J. Am. Chem. Soc. 85:2149-2154(1963))。可使用人工技术或通过自动化来进行蛋白的合成。例如，可使用 Applied Biosystems431A 肽合成器 (Perkin Elmer) 来实现自动化合成。可选择地，可分别化学合成各种片段并用化学方法组合，从而生产全长分子。

[0297] G. 组合物

[0298] 在各种实施方案中，本发明部分地包括，Wnt 多肽和编码其的多核苷酸的新组合物。如本文其他地方所述，Wnt 治疗应用的主要限制或障碍之一是其低溶解度，这使得其无法在临床规模上生产。本发明发明人设计了新的 Wnt 多肽，相比天然存在的 Wnt，其具有增加的溶解度、稳定性、生产、全身性递送和组织吸收，并保留了或具有增加的 Wnt 生物活性。在具体的实施方案中，本发明提供了可溶性 Wnt 多肽的水性制剂，以促进干细胞扩增和肌肉肥大，并促进细胞和 / 或组织形成、再生、维持和修复。

[0299] 本发明的组合物可包含一种或多种如本文所述的多肽、多核苷酸、包含其的载体等，以及一种或多种药学上可接受的盐、载体、稀释剂、赋形剂和 / 或生理上可接受的溶液，以用于单独地或与一种或多种其他治疗形式组合给予至细胞或动物。还应理解，如需要，本

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发明的组合物还可与其他试剂例如，其他蛋白、多肽、分子或各种药物活性剂组合给药。对于也可包含在所述组合物中的其他组分几乎没有限制，条件是其他药剂不会负面地影响所述 Wnt 组合物的治疗潜力，如所述组合物促进肌肉肥大以及促进组织形成、再生、维持和修复的能力。

[0300] “药学上可接受的载体、稀释剂或赋形剂”包括但不限于任何佐剂、载体、赋形剂、助流剂、增甜剂、稀释剂、防腐剂、染料 / 着色剂、风味增强剂、表面活性剂、湿润剂、分散剂、悬浮剂、稳定剂、等渗剂、溶剂或乳化剂，其被美国食品和药物管理局批准为用于人或家养动物为可接受的。

[0301] 可用作药学上可接受载体的材料的其他示例性实例包括：(1) 糖，如乳糖、葡萄糖和蔗糖；(2) 淀粉，如玉米淀粉和马铃薯淀粉；(3) 纤维素及其衍生物，如羧甲基纤维素钠、乙基纤维素和醋酸纤维素；(4) 粉状黄蓍胶；(5) 麦芽；(6) 明胶；(7) 滑石；(8) 赋形剂，如可可油和栓剂蜡；(9) 油类，如花生油、棉籽油、红花油、芝麻油、橄榄油、玉米油和大豆油；(10) 二醇类，如丙二醇；(11) 多元醇，如丙三醇、山梨醇、甘露醇和聚乙二醇；(12) 酯类，如油酸乙酯和月桂酸乙酯；(13) 琼脂；(14) 缓冲剂，如氢氧化镁和氢氧化铝；(15) 藻酸；(16) 无热源水；(17) 等渗盐水；(18) 林格氏溶液；(19) 乙醇；(20)pH 缓冲液；(21) 聚酯、聚碳酸酯和 / 或聚酐类；(22) 药学上可接受的细胞培养基；以及 (23) 用于药物制剂中的其他无毒相容物质。

[0302] 就此而言，在本发明中可使用赋形剂，用于多种目的，如调节制剂的物理、化学或生物学特性，如调节粘度和 / 或本发明的工艺过程以提高效力和 / 或使该制剂稳定，以及针对防止由于例如制造、运输、储藏、使用前准备、给药期间及此后产生的压力所造成的降解和损坏的工艺过程。

[0303] 可获得关于就此有用的蛋白稳定和制剂材料和方法的众多阐述，如 Arakawa et al.，“Solvent interactions in pharmaceutical formulations，”Pharm Res.8(3):285-91(1991);Kendrick et al.，“Physical stabilization of Proteins in aqueous solution，”RATIONAL DESIGN OF STABLE PROTEIN FORMULATIONS:THEORY AND PRACTICE,Carpenter and Manning,eds.Pharmaceutical Biotechnology.13:61-84(2002)，以及 Randolph et al.，“Surfactant-protein interactions，”Pharm Biotechnol.13:159-75(2002)，其各自通过其引用全部并入本文，尤其是与赋形剂和加工其以用于本发明的制剂相关的部分，尤其是对于蛋白药物产品和加工用于兽医和 / 或人医疗应用。

[0304] 在某些实施方案中，本发明组合物包含选自以下的赋形剂：环糊精及衍生物、纤维素、脂质体、胶束形成剂例如，胆酸，以及聚合物载体例如，聚酯和聚酐。

[0305] 在具体的实施方案中，组合物尤其是药物蛋白组合物，包含蛋白和溶剂，且进一步包含一种或多种药学上可接受的表面活性剂，优选以下一种或多种：聚山梨醇酯 20、聚山梨醇酯 80、山梨聚糖的其他脂肪酸酯、聚乙氧烯化物和泊洛沙姆 188，尤其优选聚山梨醇酯 20 或聚山梨醇酯 80，优选约 0.001%-0.1% 聚山梨醇酯 20 或聚山梨醇酯 80，非常优选约 0.002%-0.02% 聚山梨醇酯 20 或聚山梨醇酯 80，尤其是 0.002%-0.02% 聚山梨醇酯 20 或聚山梨醇酯 80。许多其他这类表面活性剂可用于本发明的实施方案中。除此以外还包括以下物质：吐温 20，包括但不限于约 0.0005% 至约 0.01% 吐温 20；胆酸钠，包括但不限

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于约 0.001% 至约 0.01% 胆酸钠；甘胆酸钠 (sodium glycholate)，包括但不限于约 0.001% 至约 0.01% 甘胆酸钠；脱氧胆酸钠，包括但不限于约 0.001% 至 0.01% 脱氧胆酸钠；甘氨脱氧胆酸钠，包括但不限于约 0.001% 至约 0.01% 甘氨脱氧胆酸钠；CHAPS，包括但不限于约 0.001% 至约 0.01%CHAPS；CHAPSO，包括但不限于约 0.001% 至约 0.01%CHAPSO；Emphigen BB，包括但不限于约 0.001% 至约 0.01%Emphigen BB；SDS，包括但不限于约 0.001% 至约 0.01%SDS；Mega-8，包括但不限于约 0.001% 至约 0.01%Mega-8；Genepol C-100，包括但不限于约 0.001% 至约 0.01%Genepol C-100；Brij35，包括但不限于约 0.001% 至约 0.01%Brij35；Pluronic F-68，包括但不限于约 0.001% 至约 0.01%Pluronic F-68；Pluronic F-127，包括但不限于约 0.001% 至约 0.01%Pluronic F-127；Zwittergent3-12，包括但不限于约 0.001% 至约 0.01%Zwittergent3-12；PEG-8000，包括但不限于约 0.001% 至约 0.01%PEG-8000；PEG-4000，包括但不限于约 0.001% 至约 0.01%PEG-4000；HPCD，包括但不限于约 0.001% 至约 0.1%HPCD；以及 Triton X-100，包括但不限于约 0.001% 至约 0.01%Triton X-100。

[0306] “药学上可接受的盐”包括酸和碱加成盐。

[0307] “药学上可接受的酸加成盐”是指保留了游离碱的生物学效力和特性的那些盐，其在生物学上或其他方面并非不利的，且其以无机酸和有机酸形成，所述无机酸诸如但不限于盐酸、氢溴酸、硫酸、硝酸、磷酸等，所述有机酸诸如但不限于乙酸、2,2- 二氯乙酸、己二酸、藻酸、抗坏血酸、天冬氨酸、苯磺酸、苯甲酸、4- 乙酰氨基苯甲酸、樟脑酸、樟脑 -10- 磺酸、羊蜡酸、己酸、辛酸、碳酸、肉桂酸、柠檬酸、环拉酸、十二烷基硫酸、乙烷 -1,2- 二磺酸、乙磺酸、2- 羟乙磺酸、甲酸、富马酸、半乳糖二酸、龙胆酸、葡庚糖酸、葡糖酸、葡糖醛酸、谷氨酸、戊二酸 (gfiitaric acid)、2- 氧 - 戊二酸、甘油磷酸、乙醇酸、马尿酸、异丁酸、乳酸、乳糖醛酸、月桂酸、马来酸、苹果酸、丙二酸、扁桃酸、甲磺酸、粘液酸、萘 -1,5- 二磺酸、萘 -2- 磺酸、1- 羟基 -2- 萘甲酸、烟酸、油酸、乳清酸、草酸、棕榈酸、双羟萘酸、丙酸、焦谷氨酸、丙酮酸、水杨酸、4- 氨基水杨酸、癸二酸、硬脂酸、琥珀酸、酒石酸、硫氰酸、甲苯磺酸、三氟乙酸、十一碳烯酸等。

[0308] “药学上可接受的碱加成盐”是指保留了游离酸的生物学效力和特性的那些盐，其在生物学上或其他方面并非不利的。这些盐是把无机碱或有机碱加入至游离酸酯而制备。衍生自无机碱的盐，包括但不限于，钠盐、钾盐、锂盐、铵盐、钙盐、镁盐、铁盐、锌盐、铜盐、锰盐、铝盐等。优选的无机盐为铵盐、钠盐、钾盐、钙盐和镁盐。衍生自有机碱的盐，包括但不限于，伯胺、仲胺和叔胺的盐、取代的胺，包括天然存在的取代的胺、环胺，以及碱性离子交换树脂，如氨、异丙胺、三甲胺、二乙胺、三乙胺、三丙胺、二乙醇胺、乙醇胺、二甲基乙醇胺、2- 二甲基氨基乙醇、2- 二乙基氨基乙醇、二环己基胺、赖氨酸、精氨酸、组氨酸、咖啡因、普鲁卡因、哈胺 (hydrabamine)、胆碱、甜菜碱、苯乙苄胺、苄星、乙二胺、葡糖胺、甲葡糖胺、可可碱、三乙醇胺、氨基丁三醇、嘌呤、哌嗪、哌啶、N- 乙基哌啶、聚胺树脂等。尤其优选的有机碱为异丙胺、二乙胺、乙醇胺、三甲胺、二环己胺、胆碱和咖啡因。

[0309] “药物组合物”是指本发明的化合物和本领域通常接受的用于将具有生物活性的化合物递送至哺乳动物例如人的媒介。这样的媒介包括用于其的所有药学上可接受的载体、稀释剂或赋形剂。

[0310] 配制组合物的其他方法为本领域技术人员所知，例如，描述于 Physicians Desk Reference, 第 62 版，Oradell,NJ:Medical Economics Co.,2008;Goodman&Gilman's The

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Pharmacological Basis of Therapeutics, 第 11 版, McGraw-Hill, 2005; Remington: The Science and Practice of Pharmacy, 第 20 版, Baltimore, MD: Lippincott Williams&Wilkins, 2000; and The Merck Index, 第 14 版, Whitehouse Station, NJ: Merck Research Laboratories, 2006 ;其各自的相关部分以引用的方式并入本文。

[0311] 在某些情况下,可取的是通过肠胃外给药来递送本文公开的组合物。如本文所用,词语“肠胃外给药”和“肠胃外给予”是指除肠内给药和局部给药以外的给药模式,通常通过注射给药,且包括但不限于 , 静脉内、肌肉内、动脉内、鞘内、囊内、眼眶内、心内、皮肤内、腹膜内、经气管、皮下、表皮下、关节内、囊下、蛛网膜下、脊柱内和胸骨内注射和输注。参见,例如,美国专利第 5,543,158 号 ;美国专利第 5,641,515 号和美国专利第 5,399,363 号 (其各自明确地通过引用以其整体并入本文)。

[0312] 在某些实施方案中,所述组合物可通过鼻内喷雾、吸入和 / 或其他气雾剂递送载体来递送。通过鼻气溶胶喷剂将基因、多核苷酸和肽组合物直接递送至肺的方法描述于,例如,美国专利第 5,756,353 号和美国专利第 5,804,212 号 (其各自明确地通过引用以其整体并入本文)。同样,使用鼻内微粒树脂 (Takenaga et al., 1998) 和溶血磷脂酰 - 甘油化合物 (美国专利第 5,725,871 号,其明确地通过引用以其整体并入本文) 递送药物也为药学领域所熟知。同样,以聚四氟乙烯载体基质形式经粘膜递送药物描述于美国专利第 5,780,045 号 (其明确地通过引用以其整体并入本文)。

[0313] H. 给药方法

[0314] 在一个实施方案中,使细胞例如,干细胞如卫星干细胞与包含一种或多种本发明的 Wnt 多肽和 / 或多核苷酸的组合物接触。还考虑了本发明的细胞可在体外、离体 (ex vivo) 或体内进行接触。在其他实施方案中,本发明的 Wnt 组合物被给予至对象。

[0315] 本发明的所述组合物可直接给予 (作为蛋白 / 多肽,或对于基因治疗在表达载体的情形下) 至对象,或离体递送至源自所述对象的细胞 (例如,在离体基因治疗中)。直接体内递送所述组合物通常通过肠胃外注射来实现,例如,皮下、腹膜内、经静脉内心肌、瘤内、瘤周,或注射至组织的间隙空间。其他给药模式包括经口和肺部给药、栓剂和经皮施用、经针和基因枪或无针注射器给药。

[0316] 本发明的组合物也可通过直接注射给予至组织如肌肉。在本发明的一些实施方案中,本发明的组合物通过将所述组合物直接注射至肌肉组织来给予,从而在注射的肌肉中预防肌肉减少,或促进注射的肌肉再生或修复,例如通过促进注射的肌肉的肌肉细胞扩增或肥大。

[0317] 通常,递送核酸用于离体和体外应用可通过如下来实现,例如,葡聚糖介导的转染、磷酸钙沉淀、聚凝胺介导的转染、原生质体融合、电穿孔、将多核苷酸包封于脂质体中、将 DNA 直接显微注射入细胞核,以及病毒如腺病毒 (与腺相关病毒) 或甲病毒介导,所有这些均为本领域所熟知。

[0318] 在某些实施方案中,优选的是使用病毒载体或其他体内多核苷酸递送技术来递送一种或多种修饰的 Wnt。在一个优选的实施方案中,所述病毒载体为非整合性载体或基于转位子载体。这可使用多种熟知方法中的任何一种来实现,如载体,包括腺病毒、逆转录病毒、慢病毒、腺相关病毒载体 (AAV),或使用其他病毒载体作为表达构建物 (包括但不限于牛痘病毒、脊髓灰质炎病毒和疱疹病毒)。

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[0319] 也可使用非病毒方法来给予本发明的多核苷酸。在一个实施方案中，多核苷酸可通过微注射直接给予至细胞，或通过注射给予至组织，如通过描述于 Dubensky et al.,(1984) 或 Benvenisty&Reshef(1986) 的技术。可以设想编码目标基因的 DNA 也可以类似的方式在体内转移并表达基因产物。

[0320] 本发明用于将裸 DNA 表达构建体转移至细胞的另一实施方案可涉及粒子轰击。该方法取决于将包覆有 DNA 的微弹加速至高速的能力，从而允许它们穿透细胞膜并进入细胞而不杀死细胞 (Klein et al.,1987)。在另一个实施方案中，通过电穿孔将多核苷酸给予至细胞。

[0321] I. 治疗方法

[0322] 本发明的包括但不限于截短的 Wnt7a 多肽、具有生物活性的 Wnt7a 多肽、Wnt7a 融合多肽的 Wnt 多肽和组合物可用于各种治疗应用。例如，本文所描述的组合物和方法可用于促进有需要的对象中的组织形成、再生、修复或维持。

[0323] 本发明的 Wnt 组合物的一些相关治疗应用包括需要通过增加肌肉大小、体积或强度来预防肌肉减少或再生减少的或损伤的肌肉组织的情况。这类情况可包括，例如，化疗或放射治疗后、肌肉损伤后，或在治疗或管理影响肌肉的疾病和病症中。在某些实施方案中，所述影响肌肉的疾病或病症可包括尿失禁、消耗性疾病（例如恶病质，其可能与诸如癌症或 AIDS 的疾病有关）、肌肉衰减或萎缩，或肌肉退行性疾病。肌肉衰减和萎缩可能与例如，肌肉减少症（包括年龄相关的肌肉减少症）、ICU 引起的虚弱、肌肉废用（例如由于昏迷无力、受伤或固定不动引起的肌肉费用）、手术引起的虚弱（例如，臀部或膝关节置换手术后）、肌肉退行性疾病（例如，肌肉萎缩）有关。这是不完全的列举。

[0324] 在某些实施方案中，本发明的多肽和组合物可用于刺激肌肉卫星细胞的对称性扩增，从而增加肌肉组织中固有卫星细胞或定向的前体细胞的比例。所述多肽和组合物也可用于促进肌肉肥大，如通过增加单根肌肉纤维的大小。因此，本发明的多肽和组合物可增加肌肉细胞的数目和肌肉细胞的大小，从而可用于例如，替换损伤的或有缺陷的组织，或预防肌肉萎缩或肌肉质量的减少，尤其是，涉及影响肌肉的疾病和病症的那些，如肌肉萎缩症、神经肌肉性和神经性退行性疾病、肌肉消耗性疾病和病症、萎缩症、心血管疾病、中风、心力衰竭、心肌梗塞、癌症、HIV 感染、AIDS 等。

[0325] 在其他实施方案中，所述组合物和方法可用于修复或再生功能失调的骨骼肌，例如，在患有肌肉退行性疾病的对象中。所述对象可能怀疑患有或处于患骨骼肌损伤、退化或萎缩的风险中。所述骨骼肌损伤可为疾病相关的或非疾病相关的。所述人对象可能患有或处于患肌肉退化或肌肉萎缩的风险中。所述肌肉退化或肌肉萎缩可全部或部分由疾病，例如 AIDS、癌症、肌肉退行性疾病，或它们的组合引起。

[0326] 肌肉萎缩的示例性实例，包括但不限于：杜氏肌肉萎缩症 (DMD)、贝克氏肌肉萎缩症 (BMD)、强直性肌肉萎缩症（也称斯坦纳特病）、肢 - 带型肌肉萎缩症、面肩胛臂肌肉萎缩症 (FSH)、先天性肌肉萎缩症、眼咽型肌肉萎缩症 (OPMD)、远端型肌肉萎缩症和埃 - 德二氏肌肉萎缩症。参见，例如，Hoffman et al.,N.Engl.J.Med.,318.1363-1368(1988); Bonnemann,C.G.et al.,Curr.Opin.Ped.,8:569-582(1996);Worton,R.,Science,270:755-756(1995);Funakoshi,M.et al.,Neuromuscul.Discord.,9(2):108-114(1999);Lim,L.E.and Campbell,K.P.,Cure.Opin.Neurol.,11(5):443-452(1998);Voit,T.,Brain

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Dev.,20(2):65-74(1998);Brown,R.H.,Annu.Rev.Med.,48:457-466(1997);Fisher,J.and Upadhyaya,M.,Neuromuscul.Disord.,7(1):55-62(1997)。

[0327] 在某些实施方案中,提供了如本文所述的组合物在制备用于促进有需要的对象的肌肉形成、维持、修复或肌肉再生的药物的应用。在具体的实施方案中,提供了如本文所述的组合物,以用于制备用于促进有需要的对象的肌肉形成、维持、修复或肌肉再生的药物。所述 Wnt 多肽可用于预防或治疗肌肉萎缩,如通过增加肌纤维的大小或数目。

[0328] 所述组合物可按有效量如治疗有效量给予。对于体内治疗人和非人对象,通常将包含有效量的一种或多种修饰的本发明 Wnt 多肽的组合物给予至所述对象。“有效量”是指能够以需要的剂量和时间段有效获得所需的治疗或预防结果的量。

[0329] 本发明 Wnt 多肽或包含其的组合物的“治疗有效量”可根据以下因素变化,如个体的疾病状态、年龄、性别和体重,以及 Wnt 多肽在个体中引起所需响应的能力。治疗有效量也是指在其中 Wnt 多肽的任何毒性或有害效果均被治疗上的有益效果超过的量。术语“治疗有效量”是指 Wnt 多肽或包含其的组合物有效“治疗”哺乳动物(例如,患者)的疾病或病症的量。

[0330] “预防有效量”是指能够以需要的剂量和时间段有效获得所需的预防结果的量。由于预防剂量是在疾病前或疾病早期阶段用于对象,通常但非必须地,所述预防有效量小于所述治疗有效量。

[0331] 在各种实施方案中,相比未处理的干细胞群体,本发明提供了增加成体干细胞如卫星干细胞的分裂对称性的方法。本文公开的方法还能促进对称的干细胞分裂而不改变干细胞分裂速率,并能促进干细胞群体的生存。所述方法可在体外、离体或体内进行。

[0332] 在具体的实施方案中,将包含一种或多种修饰的 Wnt 多肽和/或多核苷酸的组合物在体内给予至有需要的对象。如本文所用,术语“对象”包括当不限于哺乳动物,包括例如,人、非人灵长类(例如,狒狒、猩猩、猴)、小鼠、猪、牛、山羊、狗、猫、兔、大鼠、豚鼠、仓鼠、马、猴子、绵羊或其他非人哺乳动物;非哺乳动物,包括例如,非哺乳动物的脊椎动物,如鸟类(例如,鸡或鸭)或鱼,以及非哺乳动物的无脊椎动物。在优选的实施方案中,所述对象为人。需要治疗疾病或病症的对象包括,表现出这类疾病或病症症状的对象,如患有疾病或病症的那些对象以及处在患疾病或病症风险中的那些对象。

[0333] 在具体的实施方案中,在体内、离体或体外扩增卫星干细胞群体的方法包括,将所述干细胞与有效量的组合物接触,所述组合物包含截短的 Wnt7a 多肽、具有生物活性的 Wnt7a 多肽、Wnt7a 融合多肽,或者能结合至并激活 Fzd7 或编码此类 Wnt7a 多肽的多核苷酸的其直接同源物、其间接同源物或其同系物。

[0334] 撇开任何具体理论的束缚,据信增加组织中卫星细胞的数目提供了增强的组织再生潜力。

[0335] 在具体的实施方案中,干细胞在离体或体外被分离或维持并扩增,然后给予至有需要的对象。例如,可在离体或体外培养和扩增干细胞,并与有效量的本发明 Wnt 组合物接触,然后根据本领域技术人员已知的方法将其作为治疗性干细胞组合物给予至患者。在某些实施方案中,扩增的干细胞群体与治疗性 Wnt 组合物组合在一起给予至患者。

[0336] 所述促进干细胞扩增的方法可用于刺激干细胞的离体或体外扩增,从而提供适于移植或给予至有需要的对象的细胞群体。

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[0337] 在一些形式的排尿节制中，可使用本发明的组合物或方法治疗肌肉功能失调，例如，通过直接将蛋白注射至肌肉。因此，在一个实施方案中，所述方法可用于治疗尿失禁。

[0338] 在进一步的实施方案中，受损或功能失调的肌肉组织可为心肌。例如，所述受损肌肉组织可为由心血管事件如心肌梗塞或心力衰竭引起的心肌受损，其中所述靶干细胞可为心脏干细胞。根据本发明的另一个方面，提供了促进哺乳动物的心脏干细胞扩增或心脏肌肉肥大的方法，包括将有效量的如本文所述的组合物给予至所述哺乳动物。

[0339] 此外，除了将所述干细胞用于移植，干细胞或包含干细胞的组合物可用作研究工具和/或作为诊断分析或试剂盒的部分。不希望为限制性的，试剂盒可包含肌肉干细胞、一种或多种修饰的 Wnt 多肽、细胞培养物或生长培养基、细胞低温保藏培养基、一种或多种药学上可接受的递送介质、一种或多种修饰的 Wnt 多核苷酸序列或遗传构建物、用于将细胞移植或递送至有需要的对象的一种或多种装置、对使用、递送、移植、培养、低温保藏如本文所述细胞或其任何组合的指导。

[0340] 细胞扩增和/或肌肉肥大的指示物可被可定性或定量地监测，且包括，例如，总形态、总细胞数、组织学、组织化学或免疫组织化学的变化，或者特定细胞标记的存在、不存在或相对水平。细胞标记的存在、不存在或相对水平可通过以下技术来分析，例如，组织化学技术、免疫学技术、电泳、蛋白质印迹分析、FACS 分析、流式细胞术等。可选择地，可检测编码所述细胞标记蛋白的基因所表达的 mRNA 的存在，例如，采用 PCR 技术、Northern 印迹分析、使用合适的寡核苷酸探针等。

[0341] 本说明书中引用的所有出版物、专利申请和发布的专利均通过引用并入本文，如同每个单个出版物、专利申请或发布的专利均被明确地和单独地说明其通过引用并入本文。

[0342] 尽管为了清楚理解的目的，上述发明已经以说明和实例的方式相当详细地得以描述，对于本领域技术人员来说容易显而易见的是，基于本发明的教导，在不背离所附权利要求的精神或范围的情况下，可对其作出一些改变和修改。仅以说明而非限制的方式提供以下实施例。本领域技术人员可容易地理解，各种非关键性参数可被改变或修改而产生基本上相似的结果。

实施例

[0343] 实施例 1

[0344] 截短的 Wnt 蛋白的设计

[0345] Wnt 家族蛋白的长度为 300-400 个氨基酸，并含有几个包括糖基化和脂化的翻译后修饰。Wnt 蛋白的脂化给大规模重组生产、制剂 (formulation) 和可能的治疗应用带来了挑战。通常公认的是，Wnt 的脂化对于其信号转导活性是必需的，尽管该领域大多数研究的完成仅使用了一种同种型 (Wnt3a) 并仅通过使用一个 Wnt 信号转导通路 (β-连环蛋白依赖性转录激活的经典激活)(Willert et al2003)(Takada2006)。图 1 显示，半胱氨酸 (Wnt7a 的 Cys73) 和丝氨酸 (Wnt7a 的 Ser206) 的潜在脂化位点在 Wnt 家族成员之间是保守的。Wnt7a 的信号转导活性，尤其是其非经典的信号转导，甚至当推荐的脂化位点通过诱变为丙氨酸残基而被去除时，仍可维持。在 C73A 和/或 S206A 位点构建单个或两个丙氨酸替换，产生了具有适度改良的生产 (当在哺乳动物细胞组织培养中表达时) 和制剂特征的蛋

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白，同时其保留了体外和体内活性）。Wnt 蛋白被截短为保留活性的活性结构域。所述截短的 Wnt 允许较高水平的生产且为去脂化的—有助于水溶液中的制剂和稳定性。

[0346] 使用 ProteinPredict 软件程序分析了所述 Wnt7a 氨基酸序列以建立结构基序的预测模型，该软件程序能评估二级结构预测和可能的溶剂可接触性。图 2A 所示为针对人 Wnt7a 序列的预测，并突出显示了两个结构域：包含大多数 α－螺旋二级结构的 N 端结构域，以及包含大多数蛋白折叠二级结构的 C 端结构域。从 N 端结构域至 C 端结构域的过渡发生在约残基 235 至残基 265 之间。

[0347] ProteinPredict 二级结构预测程序还用于定征经典的人 Wnt3a 蛋白。图 2B 所示为 Wnt3a 可能的结构域结构，其类似于 Wnt7a 的结构，具有 N 端 α－螺旋结构和 C 端蛋白折叠结构。在人 Wnt3a 中，这两个结构域之间的过渡发生于约残基 237-270。预测的 C 端结构域不含潜在的脂化位点，如之前所绘制。构建了这些结构域的表达盒以评估是否 Wnt 信号转导活性能保留在所述 Wnt 蛋白的单个区域内，并同时使对脂质翻译后修饰的需要最小化。该蛋白可有益于蛋白生产、制剂，以及最终的治疗应用和工业应用。

[0348] 设计了一些表达构建物用于评估构建活性 Wnt 信号转导分子并同时将氨基酸序列截短为不连续的未脂化结构域的可能性。图 3 所示为突出显示各种 Wnt7a 蛋白形式的示意图。图示了潜在脂化位点的丙氨酸替换（Wnt7a C73A 和 / 或 S206A）。如以下所述，将几个截短的 Wnt 构建物置于细菌表达盒中。构建了用于在哺乳动物表达系统中生产的大多数蛋白形式。对于这些形式，内源 Wnt7a 分泌信号肽被外源信号肽如 IgG-κ 、CD33 或 IL2 信号肽替换。信号肽可潜在地改善来自哺乳动物表达系统的重组蛋白的分泌效率。

[0349] 产生以下两种不同 C 端结构域 Wnt 的截短物表达于哺乳动物组织培养物中并被检测到：Wnt7a aa235-349 和 Wnt7a aa264-349。如通过预测所评估，Wnt7a aa264-349 包含更明确的结构域，并同时保持有偶数个半胱氨酸残基（12）。Wnt7aaa264-349 蛋白表达为融合至人 IgG1Fc 结构域的蛋白，所述 Wnt 片段和所述 Fc 结构域之间的连接子区域中包含或不包含烟草蚀纹病毒（TEV）蛋白酶识别位点。该系统允许所述融合蛋白的有效表达和分泌，以及随后的在特异性 TEV 识别位点将 Wnt7a aa264-349 蛋白水解切割。使用亲和层析法清除所得的消化蛋白的 Fc 结构域、蛋白酶，以及任何残基、未消化的融合蛋白—产生小分子量 Wnt264-349 氨基酸蛋白片段的纯化的制剂。Wnt7a aa264-349 具有 11kDa 的计算分子量和约 17kDa 的观察分子量，该差异最可能缘于翻译后的糖基化。

[0350] 在本实施例中，所述 Wnt-Fc 融合蛋白包含对所述 Fc 区具有特异性的以下点突变：E233P/L234V/L235A/ΔG236+A327G/A330S/P331S。根据所述构建物，这些突变对应于 Wnt 融合蛋白中的各个位置。此外，这些突变降低了 IgG1Fc 结构域对于 Fcγ 受体的亲和力，因此，限制了所述融合蛋白的任何不良免疫激活的可能性。所有实例的序列描述和对应的序列识别如图 3 和所附的序列表文件所示。

[0351] 实施例 2

[0352] 截短的 Wnt 的构建

[0353] 根据以下方法构建截短的 Wnt 多肽和包含其的载体。

[0354] Wnt 的用于细菌表达的载体构建

[0355] 使用野生型人 Wnt7a 作为模板进行 PCR，构建了包含 Wnt7a C 端结构域的 pET29a(+) 表达载体。使用正向引物 5'-GCATCATATGGCCGTTCACGTGGAGCCTG-3'（SEQ ID

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NO:24) 和反向引物 5'-GCATGCGGCCGCTCACTTGCACGTGTACATCTCC-3' (SEQ ID NO:25) 扩增编码 Wnt7a 的氨基酸 235-349 的多核苷酸序列。PCR 产物经 NdeI 和 Not1 限制酶消化并在 NdeI 和 Not1 位点之间连接至 pET29a(+) 载体。使用PfuUltraII®聚合酶制备所述截短的 Wnt7a 构建物。

[0356] 使用野生型人 Wnt7a 作为模板进行 PCR,构建了包含 Wnt7a C 端结构域的 pET28a(+) 表达载体。使用正向引物 5'-GCATCCATGGCCGTTCACGTGGAGCCTG-3'(SEQ ID NO:26) 和反向引物 5'-GCATGCGGCCGCTCACTTGCACGTGTACATCTCC-3'(SEQ ID NO:25) 扩增编码 Wnt7a 的氨基酸 235-349 的多核苷酸序列。PCR 产物经 NcoI 和 Not1 限制酶消化并在 NcoI 和 Not1 位点之间连接至 pET28a(+) 载体。使用PfuUltraII®聚合酶制备所述截短的 Wnt7a 构建物。

[0357] 实施例 3

[0358] Wnt 融合多肽的构建

[0359] 根据以下方法构建 Wnt 融合多肽和包含其的载体。

[0360] Wnt 的用于哺乳动物表达的载体构建

[0361] 构建了包含融合至人 Wnt7a C 端结构域的 CD33 信号肽的、融合有 TEV 蛋白酶位点和 6HIS 标签的 pcDNA3.1(+) 表达载体。CD33 的多核苷酸序列 (5'-ATGCCCCTGCTGCTGCTCCTCCCTCTGCTGTGGGCTGGCGCTCTGGCCATGGAT-3' (SEQ ID NO:27)) 编码氨基酸序列 MPLLLLLPLLWAGALAMD(SEQ ID NO:28)),并融合至编码人 Wnt7a 的氨基酸 235-349 的多核苷酸序列。将所得构建物克隆至包含 TEV 蛋白酶位点和 6HIS 表位序列的 pcDNA3.1(+) 表达载体。所述融合多肽的氨基酸序列如 SEQ ID NO:10 所示。

[0362] 构建了包含融合至 Wnt7a C 端结构域的 IgGκ 信号肽的、融合有 TEV 蛋白酶位点和 FLAG 标签的 pcDNA3.1(+) 表达载体。IgGκ 的多核苷酸序列 (5'-ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGAC-3' (SEQ ID NO:29)) 编码氨基酸序列 METDTLLLWVLLLWVPGSTGD(SEQ ID NO:30)),并融合至编码人 Wnt7a 的氨基酸 235-349 的多核苷酸序列。将所得构建物克隆至包含 TEV 蛋白酶位点和 FLAG 表位序列的 pcDNA3.1(+) 表达载体。所述融合多肽的氨基酸序列如 SEQ ID NO:13 所示。

[0363] 还制备了下列 Wnt7a 构建物并将其克隆至哺乳动物细胞表达载体如 pcDNA3.1(+) :人 Wnt7a 的 Wnt7a aa31-349、Wnt7a aa235-349 和 / 或 Wnt7aaa264-349,其组合有编码氨基酸序列 MPLLLLLPLLWAGALAMD(SEQID NO:28) 的 CD33 分泌信号肽 (5'ATGCCCCTGCTGCTGCTCCTCCCTCTGCTGTGGGCTGGCGCTCTGGCCATGGAT-3'(SEQ ID NO:27)),或编码氨基酸序列 METDTLLLWVLLLWVPGSTGD(SEQ ID NO:30)) 的 IgGκ 链分泌型信号肽 (5'-ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGAC-3' (SEQ ID NO:29))。在没有任何其他标签或融合物的情况下构建这些融合蛋白,以制备 SEQ ID NO:33、34、35 和 36 所示的多肽序列。而且,通过添加 C 端 IgG-Fc 结构域,构建了同样的截短的 Wnt/ 信号肽融合物。此处所用的特定 FC- 融合结构域为人 IgG1,其具有以下突变以降低效应细胞功能 :E233P/L234V/L235A/ΔG236+A327G/A330S/P331S。这些构建物的截短的 Wnt7a-Fc- 融合多肽序列如 SEQ ID NO:37、38、39 和 40 所示。

[0364] 使用 CD33 和 IgGκ 链外源分泌信号肽并结合 Fc- 融合物构建了其他截短形式的 Wnt7a,但在所述 Wnt 和所述 Fc 结构域之间包含有蛋白酶识别位点。这些构建物利用 Fc- 融合蛋白的改良的表达和纯化,并最终从所述 Wnt 多肽移除 Fc 结构域。这些构建物的截短的

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Wnt7a-Fc 融合多肽序列如 SEQ ID NO:41、42、43 和 44 所示。

[0365] 实施例 4

[0366] Wnt 蛋白表达和纯化

[0367] 活性 Wnt 蛋白的有效、规模化生产受制于重组系统中相对低的 Wnt 蛋白表达和分泌加上这些脂化蛋白制剂的挑战的组合。已经在哺乳动物系统中小规模地有效制备活性 Wnt，并在有效地将脂化 Wnt 保持在活性构象的清洁剂和脂质体的存在下进行有效纯化 (Willert2008)(Morrel2008)。对于经典的 Wnt 蛋白的这些研究已完成，而对于非经典的 Wnt 蛋白的研究则还未完成。然而，脂质体制剂的应用对于治疗生产是个挑战，且使用清洁剂如 3-[(3- 胆酰胺丙基) 二甲氨基]-1- 丙磺酸盐 (CHAPS) 不一定适于治疗给药。制备了包含外源信号肽的全长 Wnt 蛋白，希望所述外源信号肽将改善分泌效率。此外，表达了具有外源信号肽和 Fc 结构域融合物的分泌型和纯化的全长 Wnt。还制备了如实施例 1-3 所述的表达和纯化的 Wnt 截短物，再次比较了外源信号肽和 Fc- 融合蛋白的应用。

[0368] 对于哺乳动物细胞生产，以 106 个活细胞 /ml 的细胞密度在 293Freestyle 无血清培养基中制备了 293F 悬浮培养物。在具有 1μl Mirus TransIT Pro 试剂 / μg 载体 DNA 的 Opti-MEM I 培养基中，通过 1μg Wnt 表达载体 /ml 培养物的脂质体转染对细胞进行瞬时转染。允许形成 DNA: 脂质体复合体达 25 分钟，然后加入至 293F 悬浮培养物中。将转染的细胞于 37℃、8%CO_2 下在定轨振荡器上以 130rpm 孵育 72 小时。通过两轮离心收获所述条件培养基，其中一轮以 300x g，而另一轮以 3000x g 离心，然后进行无菌过滤。使用针对 WNT 或人 IgG 的 Fc 结构域的特异性抗体，通过蛋白质印迹法定量条件培养基中的 Wnt 蛋白。根据被转染的构建物，条件培养基中正常的 WNT 产率范围为 0.9-10μg/mL。采用并行的 Sartorius Vivaflow200 装置在 2.5bar 的恒定压力下运行，通过切向流过滤将收获的培养基浓缩 5 倍。最终的培养基通过 0.2μm Millipore Opticap XL150 囊式过滤器进行无菌过滤，并继续进行蛋白纯化步骤。

[0369] 使用载入至 HiTrap Blue HP 柱 (5mL) 的澄清条件培养基纯化无 Fc- 融合物的野生型 Wnt7a 和去脂化 Wnt 蛋白。用 25mL 的 20mM Tris-HCl pH7.5、1%(w/v)CHAPS 洗柱，然后用 25mL 的 20mM Tris-HCl pH7.5、1%(w/v)CHAPS、1.5M KCl 洗脱。将用抗 Wnt7a 蛋白质印迹法检测的洗脱级分中的 Wnt7a 合并，并在 2mL 结合有抗 Wnt7a 抗体的 Sepharose4Fast Flow 上进一步纯化。以 0.2mL/min 速率进行加样，然后用 20mL PBS、1%CHAPS 洗涤。用 0.1M 甘氨酸 -HCl pH2.5、150mM NaCl、1%CHAPS 洗脱结合的 Wnt7a。以预填充有 50μL1M Tris-HCl pH9.0 的 1-mL 级分收集洗脱液。用 SDS-PAGE 分析洗脱级分中的 Wnt7a 的纯度，并用银染色检测。

[0370] 使用载入至 HiTrap rProtein A FF 柱 (5mL) 的澄清条件培养基纯化融合至人 Fc 的 Wnt7a 变体。用 40mL PBS、1%CHAPS 洗柱。用 0.1M 甘氨酸 -HCl pH2.5、150mM NaCl、1%CHAPS 洗脱结合的融合蛋白。以预填充有 0.25mL1M Tris-HCl pH9.0 的 5-mL 级分收集洗脱液。用 SDS-PAGE 分析洗脱级分中的 Wnt7a 的纯度，并用考马斯染色检测。

[0371] 将含有 Wnt7a 的级分合并，并用 Amicon Ultra-15 浓缩器浓缩至 2mL。最后使用以 PBS、1%CHAPS 平衡的 PD-10 脱盐柱 (GE Healthcare Life Sciences) 将浓缩的 Wnt7a 进行缓冲液交换。以 BSA 为标准，用 Bradford 检测测定蛋白浓度。

[0372] 以这种方式制备的 Wnt FC 融合蛋白，在表达培养基中和作为纯化后产物均清楚地

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显示了显著改良的收率（图 4）。此外，最小 Wnt 片段 -Wnt7a 氨基酸 264-349 的生产是可行的，且作为 FC- 融合蛋白产生了高收率。如通过考马斯 SDS-PAGE 所显示（图 5），这些 Wnt 蛋白还展现出高的纯度水平。

[0373] 实施例 5

[0374] Wnt 截短物保留了体外生物活性

[0375] 此前已经表明 Wnt7a 通过非经典途径（即不通过 β- 连环蛋白信号转导），最可能通过受体卷曲蛋白 7 诱导肌肉肥大和干细胞扩增 (Le Grand2009)(Von Maltzahn2012)。在培养物中野生型 Wnt7a 诱导了肌纤维肥大（图 6A）。对 Wnt7a 诱导的肌纤维肥大定量，并以图形展示于图 7。

[0376] 按如下进行体外肥大实验 :将所有细胞于 37℃、在具有 5%CO_2 的潮湿空气中培养。C2C12 细胞获自美国典型培养物保藏中心 (ATCC CRL-1772)，并维持于涂覆有明胶的组织培养平板上的、添加有 20% 胎牛血清 (FBS) 的达尔伯克氏改良伊格尔培养基 (DMEM) 中。对于体外肥大检测，将 C2C12 细胞以 2,000 细胞 / 孔接种于涂覆有明胶的 96 孔平板上。人骨骼肌成肌细胞 (HSMM) 获自 Lonza 公司，并维持于涂覆有明胶的组织培养平板上的 F10、15%FBS、0.5% 鸡胚提取物、0.4μg/ml 地塞米松和 1ng/mL 碱性成纤维细胞生长因子中。对于体外肥大检测，将 HSMM 以 12,000 细胞 / 孔接种于涂覆有明胶的 96 孔平板上。对于体外肥大检测中的 C2C12 细胞和 HSMM，24 小时后将培养基换成添加有 2% 马血清的 DMEM。3 天后加入 Wnt 蛋白，并使其与所述细胞一起再孵育 2 天。将细胞固定 (4% 多聚甲醛 PBS，10 分钟)，用 0.1%Triton X-100/PBS 进行透化，用溶于 PBS 的 10% 山羊血清和 0.1%Triton X-100 封闭，并用小鼠抗 slow MyHC 和小鼠抗 fast MyHC 染色。用 PBS 洗涤细胞，然后用山羊抗小鼠 Alexa488 染色。用 DAPI 染色细胞核。使用 Axiovision 软件进行图像采集和纤维直径测量。使用每孔最少 100 个直径计数和每个处理 2 孔的条件来评估不同 Wnt 蛋白的体外活性。

[0377] 野生型 Wnt7a(wtWnt7a) 和 Fc 融合物 (wtWnt7a-FC) 均诱导了肥大（图 7）。出人意料的是，当作为 Fc- 融合蛋白（图 7）或在从 Fc 结构域蛋白水解切割后（图 8C）使用时，Wnt7a aa264-349 也诱导了明显的肥大。较长的 C 端 Wnt7a 片段 -Wnt7a aa235-349 也在小鼠和人成肌细胞包括人原代抗肌萎缩病成肌细胞中诱导了明显的肥大（图 8A 和 8B）。这些结果清楚地表明，甚至在所述蛋白被明显截短而产生出无预测的脂化位点的片段后，仍然保留了 Wnt 活性。此外，虽然在清洁剂 CHAPS 中配制时，所有检测的 Wnt 形式均有活性，但当在无 CHAPS 的磷酸盐缓冲盐水中配制时，wtWnt7a 失去了其大多数的生物活性。当 CHAPS 被移除后，wtWnt7a-Fc 和 Wnt7a 截短物均保留有活性（图 7）。该结果显示了，缺少脂质部分的截短物的对 Fc- 融合物部分的伴侣活性和增加的水稳定性。

[0378] 实施例 6

[0379] 截短的 Wnt 蛋白和 Wnt Fc 融合蛋白具有改良的稳定性，并能够配制于治疗相关赋形剂中

[0380] 所有实施例 1-4 中设计、表达和纯化的 Wnt 蛋白在 -20℃储存和几轮冻融循环后均在肌肉肥大检测中展示了活性。当在清洁剂 CHAPS 中纯化和配制时，修饰的 Wnt 蛋白也具有活性。然而，CHAPS 并非当前普遍用于治疗性赋形剂的制剂组分。为了评估长期稳定性和在与治疗应用更相关的赋形剂中重新配制所述 Wnt 蛋白的可能性，进行了加速稳定性研

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究以及肌纤维肥大活性评估。

[0381] 于 4℃或 37℃，通过孵育相同的蛋白浓度 0、1、4 或 7 天，评估了各种 Wnt7a 蛋白形式的蛋白稳定性。评估了 3 种不同的赋形剂制剂：0.2%CHAPS/PBS、0.05% 聚山梨醇酯 80(PS80)/PBS 或仅 PBS。剩余的蛋白使用蛋白质印迹分析进行评估。用像素密度测定法将蛋白质印迹信号转换为表示剩余蛋白级分相比起始蛋白量（时间为 0）的值。当于 4℃在 CHAPS 或聚山梨醇酯制剂中孵育时，所有蛋白形式均为稳定的（图 9）。然而，当不使用清洁剂于 4℃在 PBS 中延长孵育时，大量的蛋白丢失。此外，在 PBS 中，仅 Wnt7a aa264-349 或作为 Fc- 融合物均比全长 Wnt7a-Fc 融合物明显更加稳定。该结果表明，Wnt 截短物在这些条件下是有利的。

[0382] 37℃时，在PBS中配制的所有3种蛋白制剂均丢失了蛋白。然而，截短形式的Wnt7a具有比全长蛋白 Fc- 融合物更高的稳定性。37℃时，在清洁剂存在下，在所有 Wnt7a 蛋白形式中均随时间发生了蛋白降解，但比仅 PBS 制剂的降解速度更慢。这些数据清楚地表明，Wnt7a 蛋白包括其截短物和融合物，可配制于治疗相关赋形剂如聚山梨醇酯 80 中，并保留大量的蛋白稳定性。

[0383] 在加速稳定性测试后，评估了剩余的Wnt7a蛋白活性。于4℃或37℃以相同的蛋白浓度孵育各种形式的Wnt7a蛋白0、1、4或7天。评估了赋形剂制剂0.2%CHAPS/PBS和0.05%聚山梨醇酯80。如实施例 5 和 6 中所述，评估了剩余蛋白在体外肌纤维肥大检测中的活性。使用了阴性的制剂对照和阳性的、可市购的 Wnt7a 蛋白对照。对 Wnt7a、Wnt7a-Fc- 融合蛋白、截短的 Wnt7a aa264-349 和截短的 Wnt7a aa264-349-Fc- 融合蛋白均进行了比较。

[0384] 当于 4℃在任一赋形剂中孵育达 7 天时，所有检测的蛋白形式均保留了大多数其原始的蛋白活性（图 10 和 11）。然而，当孵育温度为 37℃时，全长 Wnt7a 随时间进程而失去了其大多数活性。此外，当作为 Fc- 融合蛋白检测时，全长 Wnt7a 随时间而保留了更多活性，表明 Fc 结构域稳定了蛋白结构以及因此的活性，证实了图 7 和实施例 5 中所显示的结果。截短的 Wnt7a 片段、Wnt7a aa264-349 在两种赋形剂中均随时间进程而保留了肌肉肥大活性，并确证了截短的、非脂化 Wnt7a 蛋白形式的活性及其用于治疗性开发的改良的特性。因此，治疗相关赋形剂如聚山梨醇酯 80 可用于配制 Wnt 蛋白，包括截短的 Wnt 蛋白。

[0385] 实施例 7

[0386] Wnt7a 蛋白截短物和融合物蛋白保留了信号转导特异性

[0387] 有 19 个人 Wnt 蛋白和 10 个卷曲受体和各种共受体，如 LRP、ROR、RYK 等。已证明 Wnt7a 通过卷曲蛋白 7 受体发送信号，驱动非经典的平面细胞极性通路，并通过 G- 蛋白激活事件来激活 PI3- 激酶通路 (Von Maltzahn2012)。最充分定征的 Wnt 信号转导通路是经典的 Wnt 信号转导通路，其中 Wnt 与卷曲受体和共受体相互作用，引起 β- 连环蛋白依赖性转录激活，驱动细胞的存活、增殖以及某些情况下的分化。经改造用于治疗性开发和递送的 Wnt 蛋白应该被设计为保留了其受体和信号转导通路特异性。前述实施例中公开的经改造的截短的、非脂化 Wnt 蛋白保留了所需的肌肉肥大活性。使用相同的 Wnt7a 蛋白形式进行了进一步的试验，以排除“脱靶”效果，如激活所述经典通路的。

[0388] 为了评估这一点，我们使用了非常灵敏的报告子系统——Wnt pBAR 报告子。该报告子由连接至驱动萤火虫荧光素酶表达的最小启动子元件的 TCF 增强子元件的串联体重复组成 (Biechele2008)。当转染至哺乳动物细胞系时，该报告子可用于测量对于细胞治疗

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的经典 Wnt 活性。使用包含 pBAR 报告子的一组具有组织特异性的、已有的稳定细胞系，我们检测了用重组 Wnt3a 阳性对照或 Wnt7a 变体滴定处理所述细胞时的经典的 Wnt 信号转导。由图 12 可知，含有 pBAR 报告子的 4 个细胞系被用于检测重组 Wnt3a、全长 Wnt7a 和截短的 Wnt7a aa264-349-FC 融合物的经典 Wnt 信号转导活性。尽管 Wnt3a 在所有检测的细胞系中均诱导了强烈的荧光素酶报告子响应，Wnt7a 蛋白处理均没有引起经典的活性。因此，很显然，产生在肌肉肥大、非经典通路中保留有活性的片段的 Wnt7a 的截短没有获得经典的信号转导活性。

[0389] 实施例 8

[0390] Wnt7a 截短和融合蛋白保留了体内治疗活性

[0391] 已表明当通过注射用于体内表达的 Wnt 表达载体或纯化的蛋白制剂来直接引入至肌肉时，Wnt7a 能在啮齿类系统中诱导明显的骨骼肌肥大。体内给予 Wnt 用于人要求能够在相关的赋形剂中并以高浓度配制所述 Wnt - 以使注射量最小化。将获得了高蛋白生产水平并具有较高稳定性的 Wnt7a Fc- 融合物和 Wnt 截短物配制于治疗相关赋形剂中，并维持了体外活性。所配制的 Wnt 组合物还保留了体内活性。

[0392] 在异氟烷的麻醉下，将 Wnt7a 蛋白注射至暴露的 C57B16 小鼠左后肢胫骨前 (TA) 肌，所述小鼠具有正常肌肉功能，且抗肌萎缩病的遗传模型 C57B/scsn-DmdMdx/J 小鼠系同样如此。切口位点用外科粘合剂封闭，然后将动物保养 3 周。3 周结束后处死动物，并将 TA 肌切除、称重，并通过包埋于最适切割温度 (OCT) 包埋剂中为组织学评估做准备。将冷冻的 TA 肌以 14μm 切片，并在纯乙醇中固定 5 分钟。在 0.1%Triton-X100/PBS 中将切片透化 20 分钟。用 50:50MOM 封闭剂和 10% 山羊血清 /PBS 封闭切片，然后用抗 Pax7 抗体和 / 或抗层粘连蛋白抗体进行免疫染色。用 PBS 洗涤后，用山羊抗小鼠 Alexa555 抗体和山羊抗兔 Alexa488 抗体孵育切片。最后，用 DAPI 孵育切片，用 PBS 洗涤，并用 Fluoromount-G 封片。使用 Axiovision 软件进行图像采集，并用 Image J 软件完成用于最小菲雷特直径测量（最小纤维直径测量）的图像分析。对于每只动物，产生最少 1000 根纤维的菲雷特值，并计算中值。表示了每个处理组的中值的动物间均值，以及每个处理组的累加纤维群变化 (fiber population shift)。

[0393] 相比制剂对照注射和来自相同动物的未处理对侧肌肉，单独注射 2.5μg Wnt7a 蛋白在 C57B16 小鼠 TA 肌中诱导了明显的肌肉肥大（图 13）。所述肥大效果相当于等量的已知的肥大因子 IGF-L。通过分析来自每个处理组的所测肌纤维整体，很明显该效果是由于纤维直径中值的增加，即大多数肌肉受到影响。

[0394] 还在体内肌肉肥大检测中测定了截短的 Wnt7a aa264-349。将 2.5μgWnt7a Fc- 融合蛋白注射至抗肌萎缩病 MDX 小鼠模型的 TA 肌。3 周后，相比 Fc- 融合物对照蛋白，观察到明显的肥大（图 14）。甚至当在碱性磷酸盐缓冲盐水制剂中给予所述 Wnt 片段时，其仍诱导了肥大。因此，很明显 Wnt7a 成功地被片段化和 / 或融合至 Fc 结构域，从而提高了生产、制剂和给药参数，且仍然保留了体外和体内活性。

[0395] 实施例 9

[0396] Wnt 截短物和 Fc- 融合蛋白的改良的药代动力学特性

[0397] Wnt 是通过局部、旁分泌或梯度信号转导电位来驱动细胞过程以及组织发育和重塑的分泌蛋白。为了充分利用 Wnt 蛋白的治疗潜力，不管是作为细胞和组织再生过程的激

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动剂，或者是作为异常的营养性和肿瘤形成性生长的抑制剂，都需要相比对应的天然、未修饰的 Wnt 蛋白具有增强的全身递送潜力的蛋白形式。

[0398] 进行了药代动力学分析，以评估本发明的截短的Wnt7a和Wnt7a Fc-融合蛋白的全身递送潜力。进行了药代动力学分析，以评估所述截短的 Wnt7a 和 Wnt7a Fc- 融合蛋白的全身递送潜力。在 C57B16 小鼠中进行各种 Wnt 蛋白的快速灌注静脉注射。在 48 小时的多个时间点进行系列血液抽取。将血液采集于 EDTA 中并处理为血浆。使用夹心 ELISA 检测评估血浆样品的 Wnt7a 蛋白。针对来自所述蛋白 C 端区的肽，产生了用于检测 Wnt7a 的抗体，并预先优化以用于检测所有经改造的和截短形式的 Wnt7a 蛋白。将未修饰的多克隆抗体用作涂覆 (coating) 抗体，并将识别 Wnt7a 蛋白不同区的生物素化多克隆抗体用于检测。用未修饰的 Wnt7a 抗体涂覆平板过夜，然后用脱脂奶粉封闭。在与测试样品相同的媒介中稀释纯化的 Wnt7a 蛋白变体，并加入 (spike) 阴性对照小鼠血浆以建立标准浓度曲线。将标准物和测试样品加入至平板并孵育 1 小时。每个步骤之间都进行洗涤，将生物素化 Wnt7a 抗体加入至所述平板，然后加入缀合有中性亲和素 (neutravidin) 的辣根过氧化物酶。通过加入 TMB 试剂将 ELISA 继续 10 分钟，然后加入硫酸。在分光光度计上读取 450nm 处的吸光度，并用 Softmax 软件分析数据。进行了全身半衰期的比较评估。

[0399] 给予 50μg 全长 Wnt7a(约 2mg/kg)，并给予等摩尔量的其他 Wnt 变体。如所预期的，全长 Wnt7a 蛋白 (FTV500) 仅在第一时间点 (给予后 30 分钟) 被全身性检测到，此后则未检出 (图 15)。当作为 Fc- 融合蛋白 (FTV512) 给予时，Wnt7a 的半衰期明显提高。分子量显著增加和 Fc- 结构域通过新生 Fc 受体促进保留和循环的可能性，是有助于增加半衰期的可能因素。然而，Wnt7a-Fc 融合物的半衰期仍然相对较短。

[0400] 出人意料的是，相比全长 Fc 融合蛋白，包含氨基酸 264-349(FTV529) 的非常低分子量 (计算值 11KDa) 的 Wnt7a 截短物表现得同样好，其在 2 小时的时间点时可清楚检测到，表明这种蛋白形式更易于全身递送。相比全长 Wnt7a，表达为 Fc- 融合蛋白的 Wnt7a aa264-349 截短物显示了最显著的全身性半衰期延长，其在 30 分钟的时间点具有 6 倍大的检测，且在 8 小时的时间点时相对于背景具有清晰的检测。

[0401] 因此，本分析清楚地表明，经改造的 Wnt 蛋白具有改良的全身半衰期，且具有活性的 C 端片段 (氨基酸 264-349)Fc- 融合蛋白的性能优于其他蛋白的性能。

[0402] 一般来说，在所附权利要求中，所用术语不应解释为将权利要求限制于本说明书和权利要求中公开的具体实施方案，而应解释为包括连同这些权利要求所赋予的等效物的完整范围的所有可能的实施方案。因此，权利要求并不限于本公开。

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[0001]

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[0002]

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gcagcgagcg cacggagatg tacacgtgca agtgagcccc gtgtgcacac caccctcccg 1380

ctgcaagtca gattgctggg aggactggac cgtttccaag ctgcgggctc cctggcagga 1440

tgctgagctt gtcttttctg ctgaggaggg tacttttcct gggtttcctg caggcatccg 1500

tgggggaaaa aaaatctctc agagccctca actattctgt tccacaccca atgctgctcc 1560

accctccccc agacacagcc caggtccctc cgcggctgga gcgaagcctt ctgcagcagg 1620

aactctggac ccctgggcct catcacagca atatttaaca atttattctg ataaaaataa 1680

tattaattta tttaattaaa aagaattctt ccacaaaaaa aaaaaaaaaa aa 1732

<210> 2
<211> 349
<212> PRT
<213> 智人

<400> 2

Met Asn Arg Lys Ala Arg Arg Cys Leu Gly His Leu Phe Leu Ser Leu
1 5 10 15

Gly Met Val Tyr Leu Arg Ile Gly Gly Phe Ser Ser Val Val Ala Leu
20 25 30

Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
35 40 45

Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
50 55 60

Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65 70 75 80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu

[0003]

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85 90 95

Leu Lys Val Gly Ser Arg Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
100 105 110

Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Asn Leu
115 120 125

Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
130 135 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145 150 155 160

Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
165 170 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
180 185 190

Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
210 215 220

Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225 230 235 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
245 250 255

Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
260 265 270

Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
275 280 285

Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305 310 315 320

[0004]

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Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335

Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
340 345

<210> 3
<211> 129
<212> PRT
<213> 智人

<400> 3

Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val
1 5 10 15

His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu
20 25 30

Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu
35 40 45

Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr
50 55 60

Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln
65 70 75 80

Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His
85 90 95

Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys
100 105 110

Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys
115 120 125

Lys

<210> 4
<211> 115
<212> PRT
<213> 智人

[0005]

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<400> 4

Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr
1 5 10 15

Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr
20 25 30

Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro
35 40 45

Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala
50 55 60

Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn
65 70 75 80

Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp
85 90 95

Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr
100 105 110

Thr Cys Lys
115

<210> 5
<211> 86
<212> PRT
<213> 智人

<400> 5

Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu
1 5 10 15

Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn
20 25 30

Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg
35 40 45

Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys
50 55 60

[0006]

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Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr
65 70 75 80

Glu Met Tyr Thr Cys Lys
85

<210> 6
<211> 163
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 6

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu
20 25 30

Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr
35 40 45

Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr
50 55 60

Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro
65 70 75 80

Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala
85 90 95

Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn
100 105 110

Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp
115 120 125

Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr
130 135 140

Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly Gly Ser His His His
145 150 155 160

[0007]

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His His His

<210> 7
<211> 164
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 7

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu
20 25 30

Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr
35 40 45

Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr
50 55 60

Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro
65 70 75 80

Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala
85 90 95

Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn
100 105 110

Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp
115 120 125

Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr
130 135 140

Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly Gly Asp Tyr Lys Asp
145 150 155 160

Asp Asp Asp Lys

[0008]

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<210> 8
<211> 166
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 8

```
Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1               5                  10                  15

Gly Ser Thr Gly Asp Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys
            20                  25                  30

Tyr Asn Glu Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys
        35                  40                  45

Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro
    50                  55                  60

Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu
65                  70                  75                  80

Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn
                85                  90                  95

Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg
            100                 105                 110

Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys
        115                 120                 125

Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr
    130                 135                 140

Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly Gly Ser
145                 150                 155                 160

His His His His His His
                165
```

[0009]

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<210> 9
<211> 167
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 9

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys
20 25 30

Tyr Asn Glu Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys
35 40 45

Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro
50 55 60

Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu
65 70 75 80

Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn
85 90 95

Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg
100 105 110

Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys
115 120 125

Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr
130 135 140

Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly Gly Asp
145 150 155 160

Tyr Lys Asp Asp Asp Asp Lys
165

<210> 10
<211> 149
<212> PRT

[0010]

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<213> 人工序列

<220>
<223> 融合蛋白

<400> 10

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg
20 25 30

Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met
35 40 45

Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu
50 55 60

Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys
65 70 75 80

Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly
85 90 95

Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe
100 105 110

His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu
115 120 125

Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly Gly Ser His
130 135 140

His His His His His
145

<210> 11
<211> 150
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 11

[0011]

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Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg
20 25 30

Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met
35 40 45

Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu
50 55 60

Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys
65 70 75 80

Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly
85 90 95

Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe
100 105 110

His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu
115 120 125

Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly Gly Asp Tyr
130 135 140

Lys Asp Asp Asp Asp Lys
145 150

<210> 12
<211> 152
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 12

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg
20 25 30

[0012]

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Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg
35 40 45

Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
50 55 60

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
65 70 75 80

Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
85 90 95

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
100 105 110

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
115 120 125

Arg Thr Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly
130 135 140

Gly Ser His His His His His His
145 150

<210> 13
<211> 153
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 13

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg
20 25 30

Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg
35 40 45

Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
50 55 60

[0013]

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Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
65 70 75 80

Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
85 90 95

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
100 105 110

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
115 120 125

Arg Thr Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly
130 135 140

Gly Asp Tyr Lys Asp Asp Asp Asp Lys
145 150

<210> 14
<211> 120
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 14

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
20 25 30

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
35 40 45

Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
50 55 60

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
65 70 75 80

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu

[0014]

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85 90 95

Arg Thr Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly
100 105 110

Gly Ser His His His His His His
115 120

<210> 15
<211> 121
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 15

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
20 25 30

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
35 40 45

Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
50 55 60

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
65 70 75 80

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
85 90 95

Arg Thr Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe Gln Gly Gly
100 105 110

Gly Asp Tyr Lys Asp Asp Asp Asp Lys
115 120

<210> 16
<211> 123
<212> PRT
<213> 人工序列

[0015]

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<220>
<223> 融合蛋白

<400> 16

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser
20 25 30

Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln
35 40 45

Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu
50 55 60

Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp
65 70 75 80

Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr
85 90 95

Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe
100 105 110

Gln Gly Gly Gly Ser His His His His His His
115 120

<210> 17
<211> 124
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 17

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser
20 25 30

Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln

[0016]

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35 40 45

Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu
50 55 60

Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp
65 70 75 80

Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr
85 90 95

Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys Glu Asn Leu Tyr Phe
100 105 110

Gln Gly Gly Gly Asp Tyr Lys Asp Asp Asp Asp Lys
115 120

<210> 18
<211> 349
<212> PRT
<213> 小家鼠(Mus musculus)

<400> 18

Met Thr Arg Lys Ala Arg Arg Cys Leu Gly His Leu Phe Leu Ser Leu
1 5 10 15

Gly Ile Val Tyr Leu Arg Ile Gly Gly Phe Ser Ser Val Val Ala Leu
20 25 30

Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
35 40 45

Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
50 55 60

Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65 70 75 80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
85 90 95

Leu Lys Val Gly Ser Arg Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
100 105 110

[0017]

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Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Asn Leu
115 120 125

Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
130 135 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145 150 155 160

Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
165 170 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
180 185 190

Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
210 215 220

Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225 230 235 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
245 250 255

Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
260 265 270

Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
275 280 285

Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305 310 315 320

Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335

[0018]

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Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
340 345

<210> 19
<211> 349
<212> PRT
<213> 褐家鼠(Rattus norvegicus)

<400> 19

Met Thr Arg Lys Ala Arg Arg Cys Leu Gly His Leu Phe Leu Ser Leu
1 5 10 15

Gly Ile Val Tyr Leu Arg Ile Gly Asp Phe Ser Ser Val Val Ala Leu
20 25 30

Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
35 40 45

Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
50 55 60

Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65 70 75 80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
85 90 95

Leu Lys Val Gly Ser Arg Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
100 105 110

Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Asn Leu
115 120 125

Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
130 135 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145 150 155 160

Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
165 170 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
180 185 190

[0019]

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Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
210 215 220

Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225 230 235 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
245 250 255

Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
260 265 270

Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
275 280 285

Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305 310 315 320

Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335

Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
340 345

<210> 20
<211> 349
<212> PRT
<213> 原鸡(Gallus gallus)

<400> 20

Met Asn Arg Lys Thr Arg Arg Trp Ile Phe His Ile Phe Leu Ser Leu
1 5 10 15

Gly Ile Val Tyr Ile Lys Ile Gly Gly Phe Ser Ser Val Val Ala Leu
20 25 30

[0020]

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```
Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
        35                  40                  45

Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
    50                  55                  60

Gly Ser Gln Met Gly Ile Asn Glu Cys Gln Phe Gln Phe Arg Asn Gly
65                  70                  75                  80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
                85                  90                  95

Leu Lys Val Gly Ser Arg Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
            100                 105                 110

Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Asn Leu
        115                 120                 125

Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Lys Glu
    130                 135                 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145                 150                 155                 160

Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
                165                 170                 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
            180                 185                 190

Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
        195                 200                 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Lys Phe Arg Glu Leu
    210                 215                 220

Gly Tyr Ile Leu Lys Asp Lys Tyr Asn Glu Ala Val Gln Val Glu Pro
225                 230                 235                 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
                245                 250                 255

Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
```

[0021]

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260 265 270

Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
275 280 285

Thr Gln Gly Arg Met Cys Asn Lys Thr Ala Gln Gln Ser Asn Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ser Arg
305 310 315 320

Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335

Asn Thr Cys Ser Glu Arg Thr Glu Val Tyr Thr Cys Lys
340 345

<210> 21
<211> 349
<212> PRT
<213> 斑马鱼(Danio rerio)

<400> 21

Met Ser Arg Lys Thr Arg Arg Trp Ile Phe His Ile Phe Leu Cys Leu
1 5 10 15

Gly Ile Ile Tyr Leu Lys Ile Gly Gly Phe Ser Ser Val Val Ala Leu
20 25 30

Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
35 40 45

Arg Thr Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
50 55 60

Gly Ala Gln Met Gly Ile Asn Glu Cys Gln Phe Gln Phe Lys Asn Gly
65 70 75 80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
85 90 95

Leu Lys Val Gly Ser Lys Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
100 105 110

[0022]

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Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Thr Leu
115 120 125

Ser Gly Cys Gly Cys Asp Lys Glu Lys Gln Gly Phe Tyr Asn Gln Glu
130 135 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Leu
145 150 155 160

Ser Phe Ser Lys Val Phe Leu Asp Ala Arg Glu Ile Lys Gln Asn Ala
165 170 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Val Gly Arg Lys Ile Leu
180 185 190

Glu Lys Asn Met Arg Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Lys Phe Arg Gln Leu
210 215 220

Gly Tyr Ile Leu Lys Glu Arg Tyr Asn His Ala Val His Val Glu Pro
225 230 235 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Ala Phe Leu Lys Val Lys Lys
245 250 255

Pro Tyr Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
260 265 270

Lys Ser Pro Asn Tyr Cys Glu Ala Asp Pro Val Thr Gly Ser Met Gly
275 280 285

Thr Gln Gly Arg Ile Cys Asn Lys Thr Ala Gln His Thr Asn Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ser Arg
305 310 315 320

Val Trp Gln Cys Asn Cys Lys Phe Leu Trp Cys Cys Tyr Val Lys Cys
325 330 335

[0023]

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Asn Thr Cys Ser Glu Arg Thr Glu Val Tyr Thr Cys Lys
340 345

<210> 22
<211> 349
<212> PRT
<213> 野猪(Sus scrofa)

<400> 22

Met Asn Arg Lys Ala Arg Arg Cys Leu Gly His Leu Phe Leu Ser Leu
1 5 10 15

Gly Leu Val Tyr Leu Arg Ile Gly Gly Phe Ser Ser Val Val Ala Leu
20 25 30

Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
35 40 45

Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
50 55 60

Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65 70 75 80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
85 90 95

Leu Lys Val Gly Ser Arg Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
100 105 110

Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Asn Leu
115 120 125

Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
130 135 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145 150 155 160

Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
165 170 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
180 185 190

[0024]

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Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
210 215 220

Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225 230 235 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys
245 250 255

Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu
260 265 270

Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly
275 280 285

Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305 310 315 320

Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335

Asn Thr Cys Ser Glu Arg Thr Glu Val Tyr Thr Cys Lys
340 345

<210> 23
<211> 349
<212> PRT
<213> 牛(Bos taurus)

<400> 23

Met Asn Arg Lys Ala Arg Arg Cys Leu Gly His Leu Phe Leu Ser Leu
1 5 10 15

Gly Met Val Tyr Leu Arg Ile Gly Gly Phe Ser Ser Val Val Ala Leu
20 25 30

[0025]

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Gly Ala Ser Ile Ile Cys Asn Lys Ile Pro Gly Leu Ala Pro Arg Gln
35 40 45

Arg Ala Ile Cys Gln Ser Arg Pro Asp Ala Ile Ile Val Ile Gly Glu
50 55 60

Gly Ser Gln Met Gly Leu Asp Glu Cys Gln Phe Gln Phe Arg Asn Gly
65 70 75 80

Arg Trp Asn Cys Ser Ala Leu Gly Glu Arg Thr Val Phe Gly Lys Glu
85 90 95

Leu Lys Val Gly Ser Arg Glu Ala Ala Phe Thr Tyr Ala Ile Ile Ala
100 105 110

Ala Gly Val Ala His Ala Ile Thr Ala Ala Cys Thr Gln Gly Asn Leu
115 120 125

Ser Asp Cys Gly Cys Asp Lys Glu Lys Gln Gly Gln Tyr His Arg Asp
130 135 140

Glu Gly Trp Lys Trp Gly Gly Cys Ser Ala Asp Ile Arg Tyr Gly Ile
145 150 155 160

Gly Phe Ala Lys Val Phe Val Asp Ala Arg Glu Ile Lys Gln Asn Ala
165 170 175

Arg Thr Leu Met Asn Leu His Asn Asn Glu Ala Gly Arg Lys Ile Leu
180 185 190

Glu Glu Asn Met Lys Leu Glu Cys Lys Cys His Gly Val Ser Gly Ser
195 200 205

Cys Thr Thr Lys Thr Cys Trp Thr Thr Leu Pro Gln Phe Arg Glu Leu
210 215 220

Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu Ala Val His Val Glu Pro
225 230 235 240

Val Arg Ala Ser Arg Asn Lys Arg Pro Ala Phe Leu Lys Ile Lys Lys
245 250 255

Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Glu Leu Val Tyr Ile Glu

[0026]

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260 265 270

Lys Ser Pro Ser Tyr Cys Glu Glu Asp Pro Ala Thr Gly Ser Val Gly
275 280 285

Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys
290 295 300

Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg
305 310 315 320

Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys
325 330 335

Asn Thr Cys Ser Glu Arg Thr Glu Val Tyr Thr Cys Lys
340 345

<210> 24
<211> 29
<212> DNA
<213> 人工序列

<220>
<223> 用于构建Wnt表达载体的多核苷酸序列

<400> 24
gcatcatatg gccgttcacg tggagcctg 29

<210> 25
<211> 34
<212> PRT
<213> 人工序列

<220>
<223> 用于构建Wnt表达载体的多核苷酸序列

<400> 25

gcatgcggccgctcacttgcacgtgtacatctcc

<210> 26
<211> 28
<212> DNA
<213> 人工序列

<220>
<223> 用于构建Wnt表达载体的多核苷酸序列

[0027]

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<400> 26
gcatccatgg ccgttcacgt ggagcctg 28

<210> 27
<211> 54
<212> DNA
<213> 智人

<400> 27
atgcccctgc tgctgctcct ccctctgctg tgggctggcg ctctggccat ggat 54

<210> 28
<211> 18
<212> PRT
<213> 智人

<400> 28

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp

<210> 29
<211> 63
<212> DNA
<213> 智人

<400> 29
atggagacag acacactcct gctatgggta ctgctgctct gggttccagg ttccactggt 60

gac 63

<210> 30
<211> 21
<212> PRT
<213> 智人

<400> 30

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp
20

<210> 31
<211> 147
<212> PRT

[0028]

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<213> 人工序列

<220>
<223> 融合蛋白

<400> 31

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys Tyr Asn Glu
20 25 30

Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr
35 40 45

Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr
50 55 60

Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro
65 70 75 80

Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala
85 90 95

Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn
100 105 110

Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp
115 120 125

Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr
130 135 140

Thr Cys Lys
145

<210> 32
<211> 150
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 32

[0029]

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Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Phe Arg Glu Leu Gly Tyr Val Leu Lys Asp Lys
20 25 30

Tyr Asn Glu Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys
35 40 45

Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro
50 55 60

Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu
65 70 75 80

Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn
85 90 95

Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg
100 105 110

Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys
115 120 125

Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr
130 135 140

Glu Met Tyr Thr Cys Lys
145 150

<210> 33
<211> 133
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 33

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg
20 25 30

[0030]

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Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met
35 40 45

Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu
50 55 60

Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys
65 70 75 80

Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly
85 90 95

Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe
100 105 110

His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu
115 120 125

Met Tyr Thr Cys Lys
130

<210> 34
<211> 136
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 34

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg
20 25 30

Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg
35 40 45

Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
50 55 60

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
65 70 75 80

[0031]

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Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
85 90 95

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
100 105 110

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
115 120 125

Arg Thr Glu Met Tyr Thr Cys Lys
130 135

<210> 35
<211> 104
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 35

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
20 25 30

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
35 40 45

Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
50 55 60

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
65 70 75 80

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
85 90 95

Arg Thr Glu Met Tyr Thr Cys Lys
100

<210> 36

[0032]

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<211> 107
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 36

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser
20 25 30

Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln
35 40 45

Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu
50 55 60

Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp
65 70 75 80

Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr
85 90 95

Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
100 105

<210> 37
<211> 376
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 37

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg
20 25 30

Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met
35 40 45

[0033]

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Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu
50 55 60

Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys
65 70 75 80

Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly
85 90 95

Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe
100 105 110

His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu
115 120 125

Met Tyr Thr Cys Lys Gly Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
130 135 140

Ser Gly Gly Gly Gly Ser Asp Lys Thr His Thr Cys Pro Pro Cys Pro
145 150 155 160

Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
165 170 175

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
180 185 190

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
195 200 205

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
210 215 220

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
225 230 235 240

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly
245 250 255

Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
260 265 270

[0034]

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Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr
275 280 285

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
290 295 300

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
305 310 315 320

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
325 330 335

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
340 345 350

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
355 360 365

Ser Leu Ser Leu Ser Pro Gly Lys
370 375

<210> 38
<211> 379
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 38

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Ala Val His Val Glu Pro Val Arg Ala Ser Arg
20 25 30

Asn Lys Arg Pro Thr Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg
35 40 45

Lys Pro Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
50 55 60

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
65 70 75 80

[0035]

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Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
85 90 95

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
100 105 110

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
115 120 125

Arg Thr Glu Met Tyr Thr Cys Lys Gly Thr Gly Gly Gly Gly Ser Gly
130 135 140

Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Lys Thr His Thr Cys Pro
145 150 155 160

Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro
165 170 175

Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
180 185 190

Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
195 200 205

Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
210 215 220

Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
225 230 235 240

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
245 250 255

Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys
260 265 270

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu
275 280 285

Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
290 295 300

[0036]

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Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
305 310 315 320

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
325 330 335

Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
340 345 350

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
355 360 365

Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
370 375

<210> 39
<211> 347
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 39

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr
20 25 30

Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala
35 40 45

Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys
50 55 60

Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn
65 70 75 80

Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu
85 90 95

Arg Thr Glu Met Tyr Thr Cys Lys Gly Thr Gly Gly Gly Gly Ser Gly
100 105 110

[0037]

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Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Lys Thr His Thr Cys Pro
115 120 125

Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro
130 135 140

Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
145 150 155 160

Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
165 170 175

Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
180 185 190

Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
195 200 205

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
210 215 220

Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys
225 230 235 240

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu
245 250 255

Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
260 265 270

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
275 280 285

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
290 295 300

Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
305 310 315 320

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
325 330 335

[0038]

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Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
340 345

<210> 40
<211> 350
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 40

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser
20 25 30

Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln
35 40 45

Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu
50 55 60

Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp
65 70 75 80

Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr
85 90 95

Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys Gly Thr Gly Gly Gly
100 105 110

Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Lys Thr His
115 120 125

Thr Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe
130 135 140

Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
145 150 155 160

Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
165 170 175

[0039]

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Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
180 185 190

Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
195 200 205

Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
210 215 220

Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser
225 230 235 240

Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
245 250 255

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
260 265 270

Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
275 280 285

Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
290 295 300

Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
305 310 315 320

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
325 330 335

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
340 345 350

<210> 41
<211> 383
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 41

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala

[0040]

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1 5 10 15

Met Asp Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Pro Val
20 25 30

Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
35 40 45

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
50 55 60

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
65 70 75 80

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
85 90 95

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
100 105 110

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser
115 120 125

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
130 135 140

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
145 150 155 160

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
165 170 175

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
180 185 190

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
195 200 205

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
210 215 220

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
225 230 235 240

[0041]

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Ser Pro Gly Lys Gly Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
245 250 255

Gly Gly Gly Gly Ser Glu Asn Leu Tyr Phe Gln Gly Ala Val His Val
260 265 270

Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr Phe Leu Lys Ile
275 280 285

Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr Asp Leu Val Tyr
290 295 300

Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val Thr Gly Ser
305 310 315 320

Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro Gln Ala Ser
325 330 335

Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr His Gln Tyr
340 345 350

Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys Cys Tyr Val
355 360 365

Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr Cys Lys
370 375 380

<210> 42
<211> 387
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 42

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Ala Asp Lys Thr His Thr Cys Pro Pro Cys Pro
20 25 30

Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro

[0042]

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35 40 45

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
50 55 60

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
65 70 75 80

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
85 90 95

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
100 105 110

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly
115 120 125

Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
130 135 140

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr
145 150 155 160

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
165 170 175

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
180 185 190

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
195 200 205

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
210 215 220

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
225 230 235 240

Ser Leu Ser Leu Ser Pro Gly Lys Gly Thr Gly Gly Gly Gly Ser Gly
245 250 255

Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Asn Leu Tyr Phe Gln Gly
260 265 270

[0043]

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Ala Val His Val Glu Pro Val Arg Ala Ser Arg Asn Lys Arg Pro Thr
275 280 285

Phe Leu Lys Ile Lys Lys Pro Leu Ser Tyr Arg Lys Pro Met Asp Thr
290 295 300

Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro
305 310 315 320

Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala
325 330 335

Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn
340 345 350

Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp
355 360 365

Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr
370 375 380

Thr Cys Lys
385

<210> 43
<211> 354
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 43

Met Pro Leu Leu Leu Leu Leu Pro Leu Leu Trp Ala Gly Ala Leu Ala
1 5 10 15

Met Asp Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Pro Val
20 25 30

Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
35 40 45

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser

[0044]

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50 55 60

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
65 70 75 80

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
85 90 95

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
100 105 110

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser
115 120 125

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
130 135 140

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
145 150 155 160

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
165 170 175

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
180 185 190

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
195 200 205

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
210 215 220

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
225 230 235 240

Ser Pro Gly Lys Gly Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
245 250 255

Gly Gly Gly Gly Ser Glu Asn Leu Tyr Phe Gln Gly Met Asp Thr Asp
260 265 270

Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu Glu Asp Pro Val
275 280 285

[0045]

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Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn Lys Thr Ala Pro
290 295 300

Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg Gly Tyr Asn Thr
305 310 315 320

His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys Phe His Trp Cys
325 330 335

Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr Glu Met Tyr Thr
340 345 350

Cys Lys

<210> 44
<211> 358
<212> PRT
<213> 人工序列

<220>
<223> 融合蛋白

<400> 44

Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1 5 10 15

Gly Ser Thr Gly Asp Ala Asp Lys Thr His Thr Cys Pro Pro Cys Pro
20 25 30

Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
35 40 45

Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
50 55 60

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
65 70 75 80

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
85 90 95

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln

[0046]

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100 105 110

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly
115 120 125

Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
130 135 140

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr
145 150 155 160

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
165 170 175

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
180 185 190

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
195 200 205

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
210 215 220

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
225 230 235 240

Ser Leu Ser Leu Ser Pro Gly Lys Gly Thr Gly Gly Gly Gly Ser Gly
245 250 255

Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Asn Leu Tyr Phe Gln Gly
260 265 270

Met Asp Thr Asp Leu Val Tyr Ile Glu Lys Ser Pro Asn Tyr Cys Glu
275 280 285

Glu Asp Pro Val Thr Gly Ser Val Gly Thr Gln Gly Arg Ala Cys Asn
290 295 300

Lys Thr Ala Pro Gln Ala Ser Gly Cys Asp Leu Met Cys Cys Gly Arg
305 310 315 320

Gly Tyr Asn Thr His Gln Tyr Ala Arg Val Trp Gln Cys Asn Cys Lys
325 330 335

[0047]

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Phe His Trp Cys Cys Tyr Val Lys Cys Asn Thr Cys Ser Glu Arg Thr
340 345 350

Glu Met Tyr Thr Cys Lys
355

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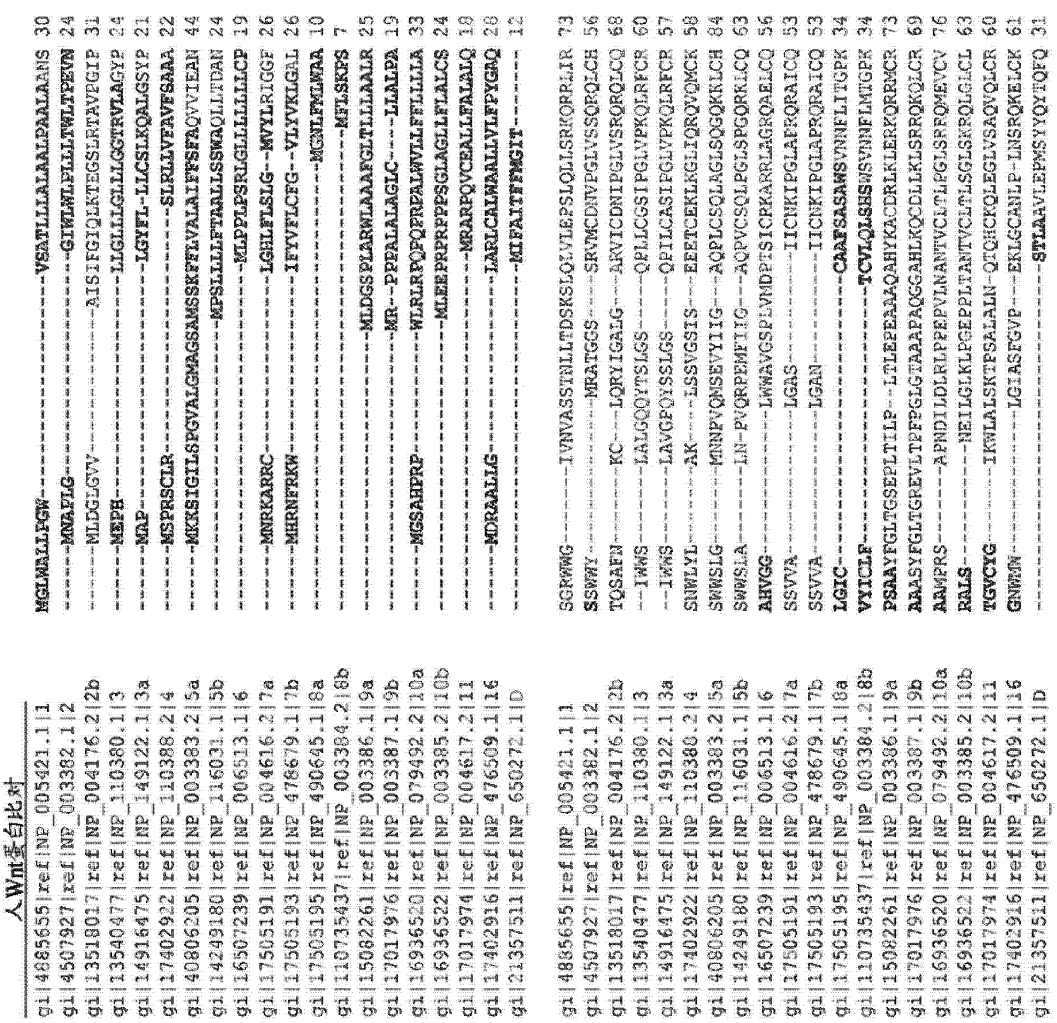


图 1

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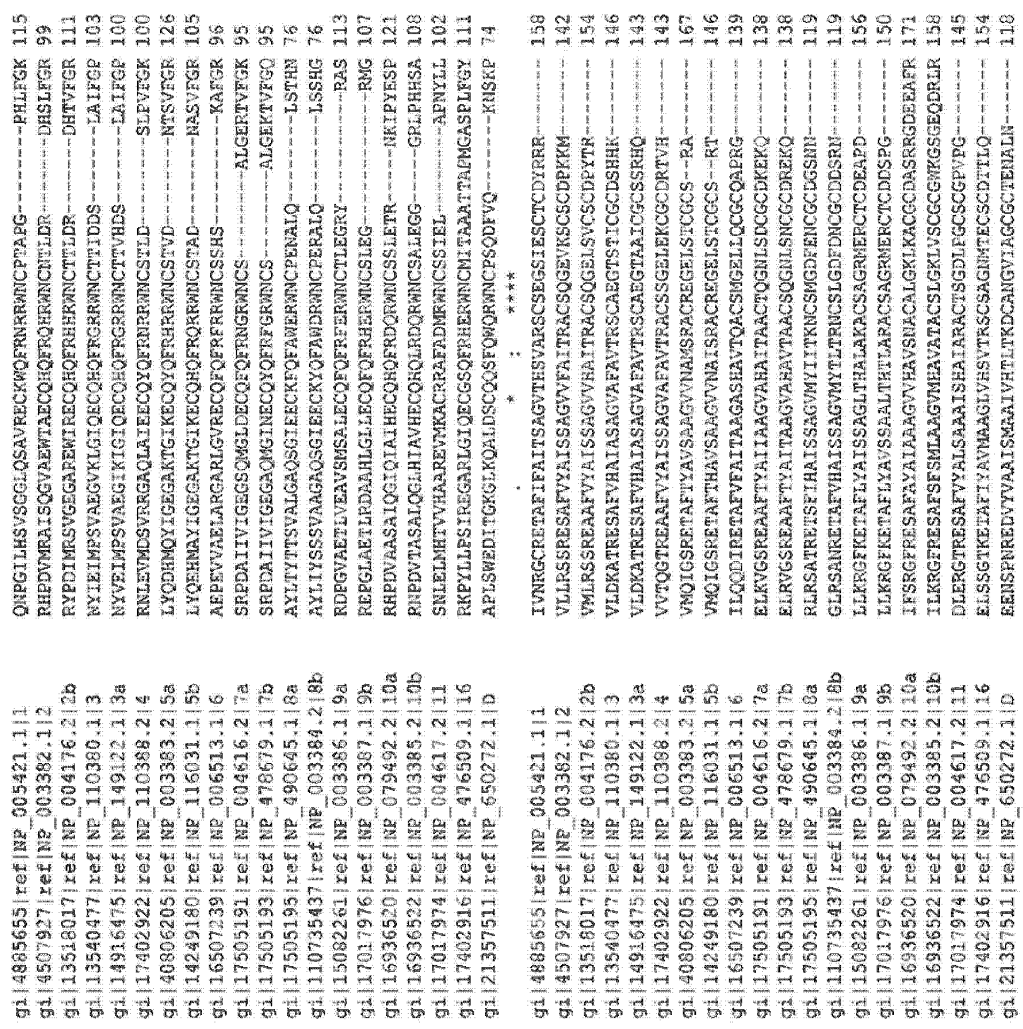


图 1（续）

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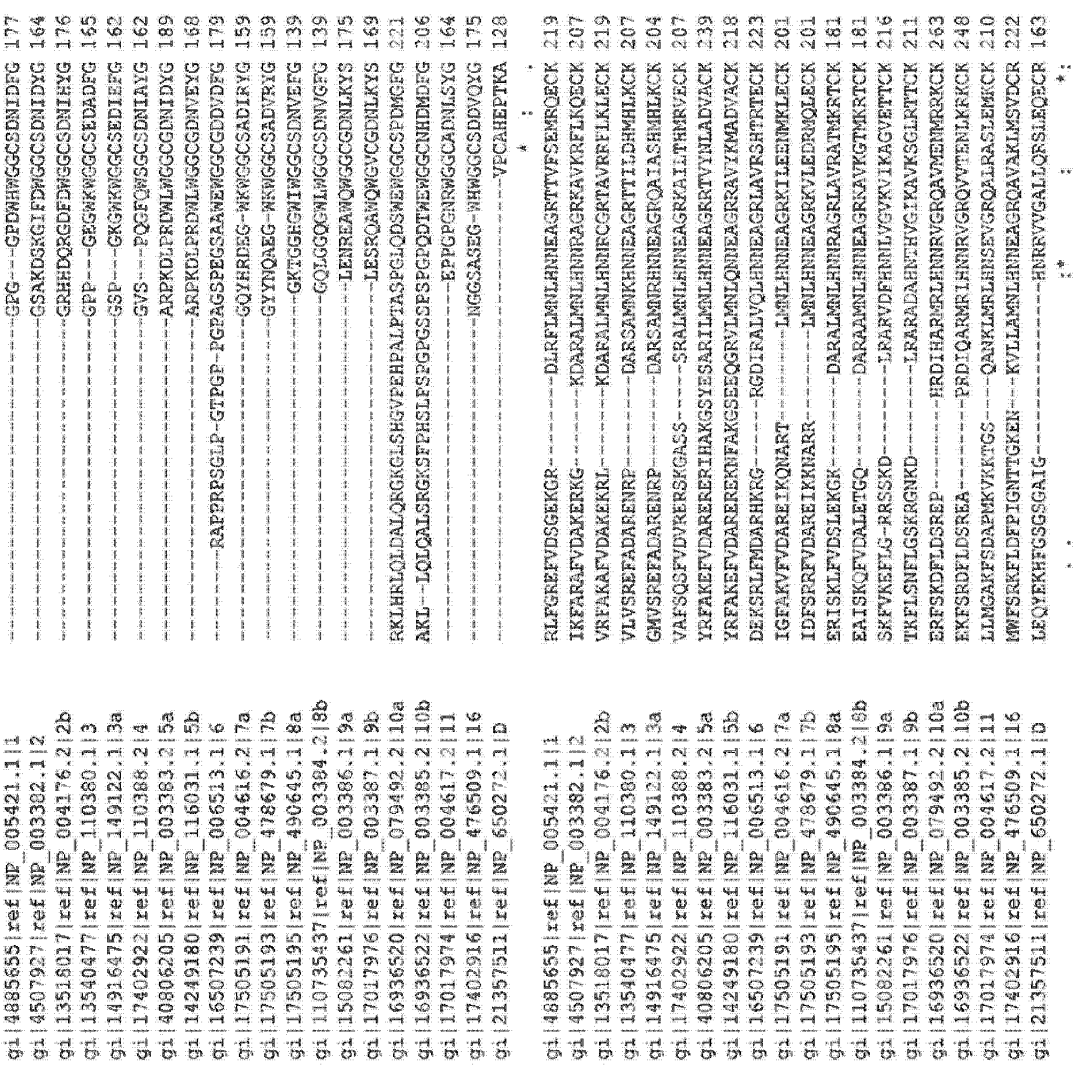


图 1（续）

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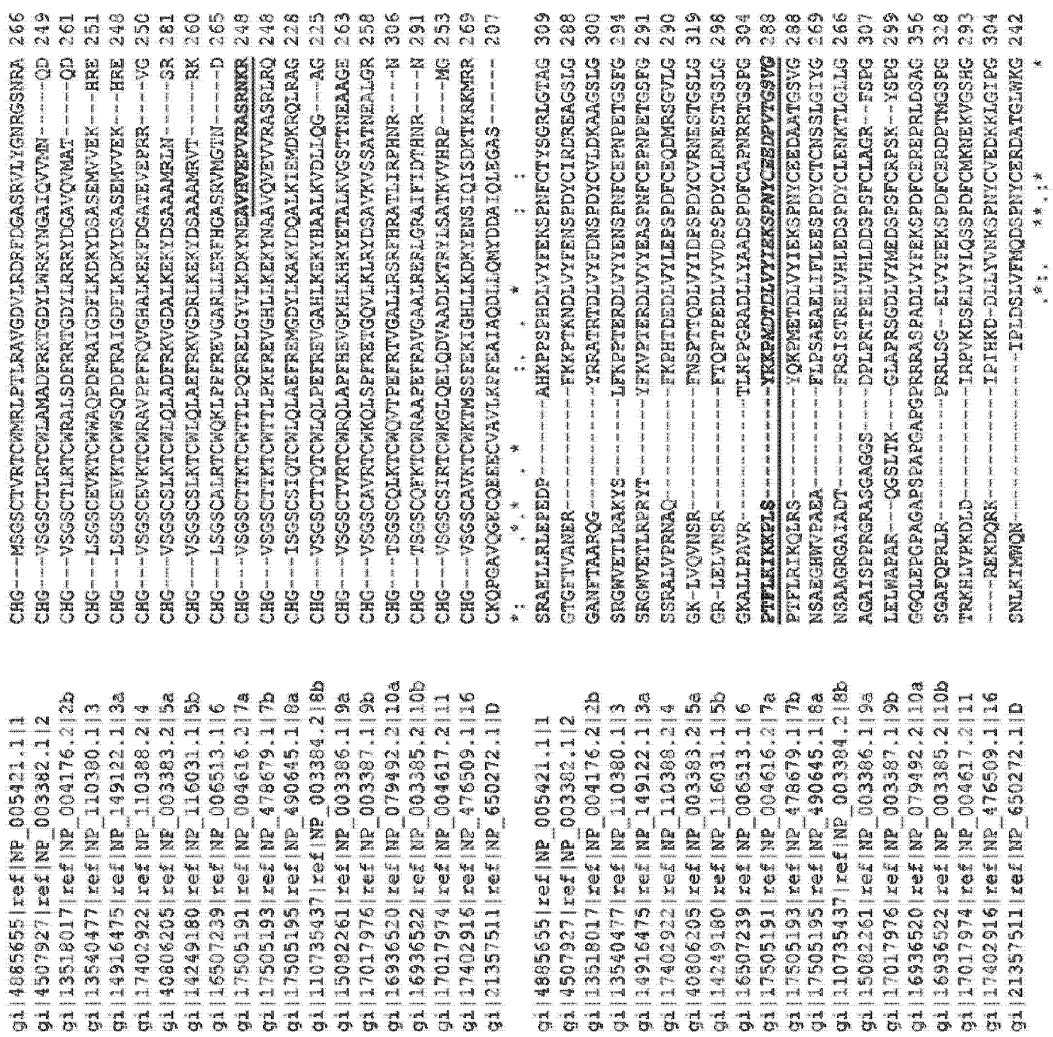


图 1（续）

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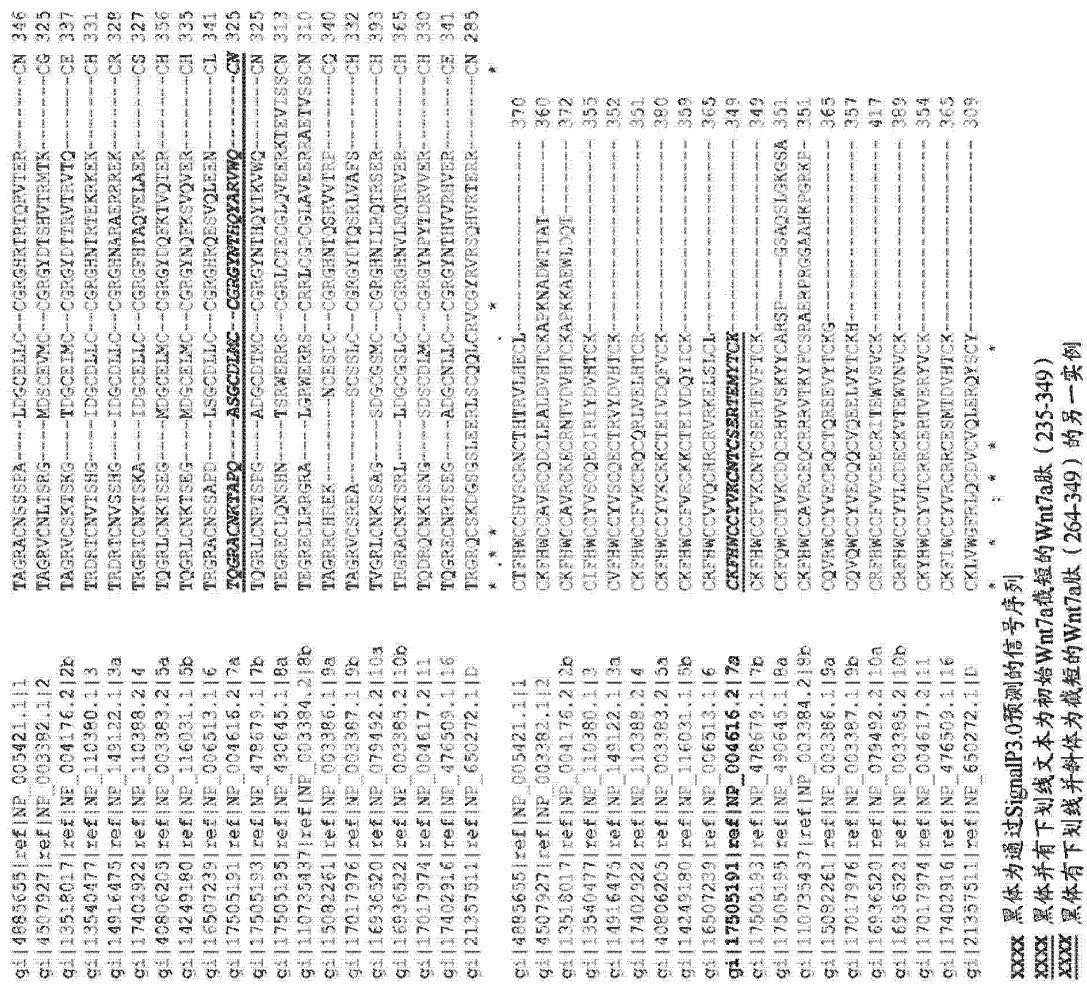


图 1（续）

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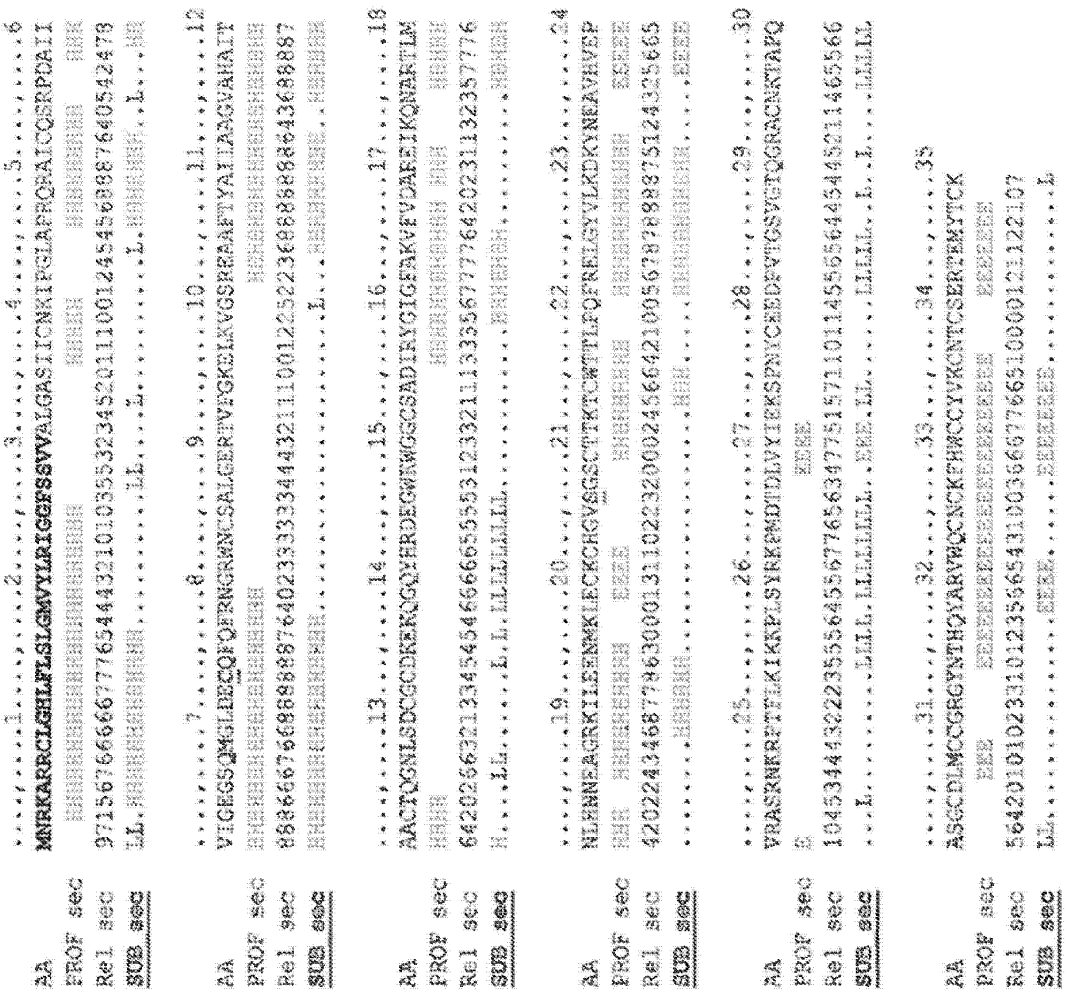


图 2A

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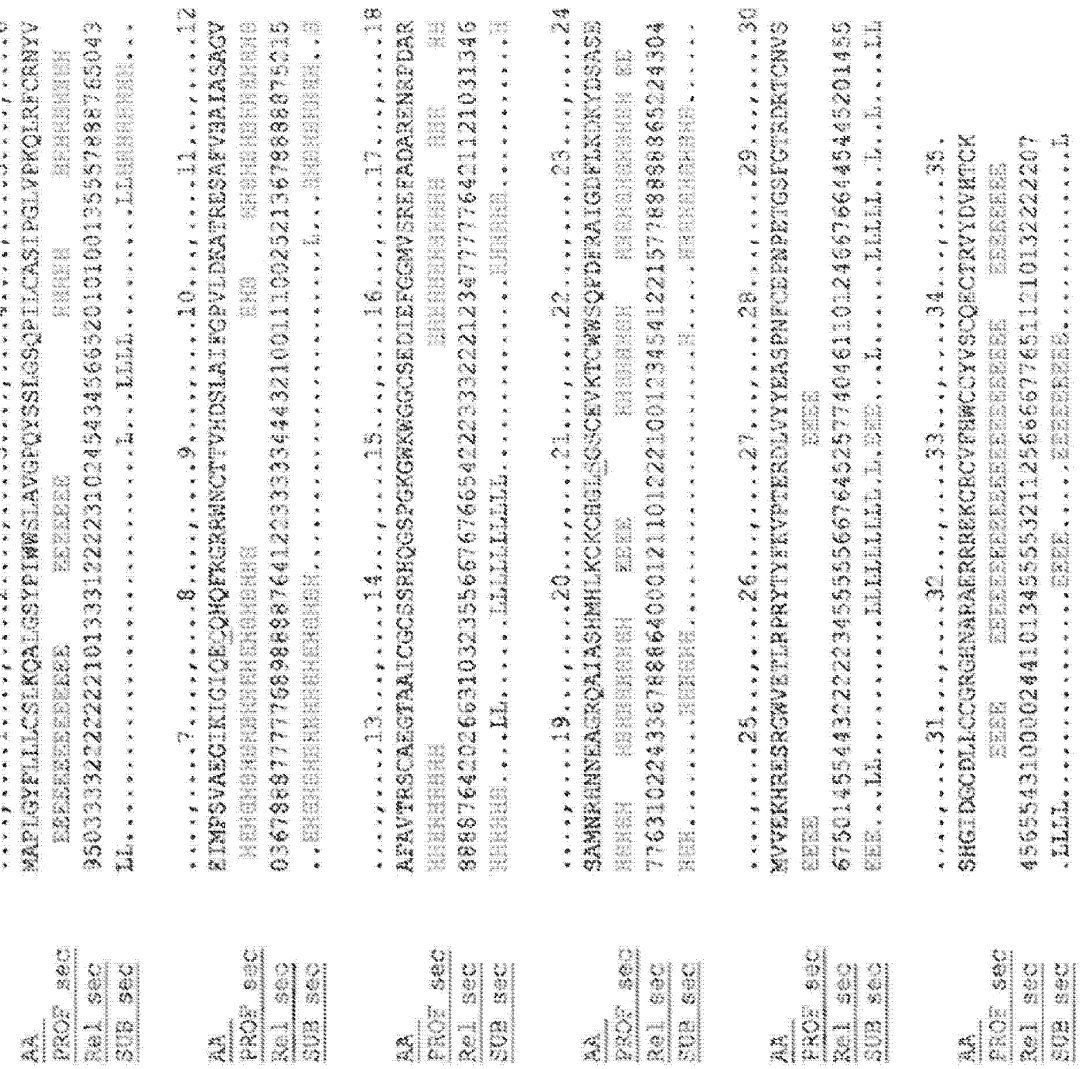


图 2B

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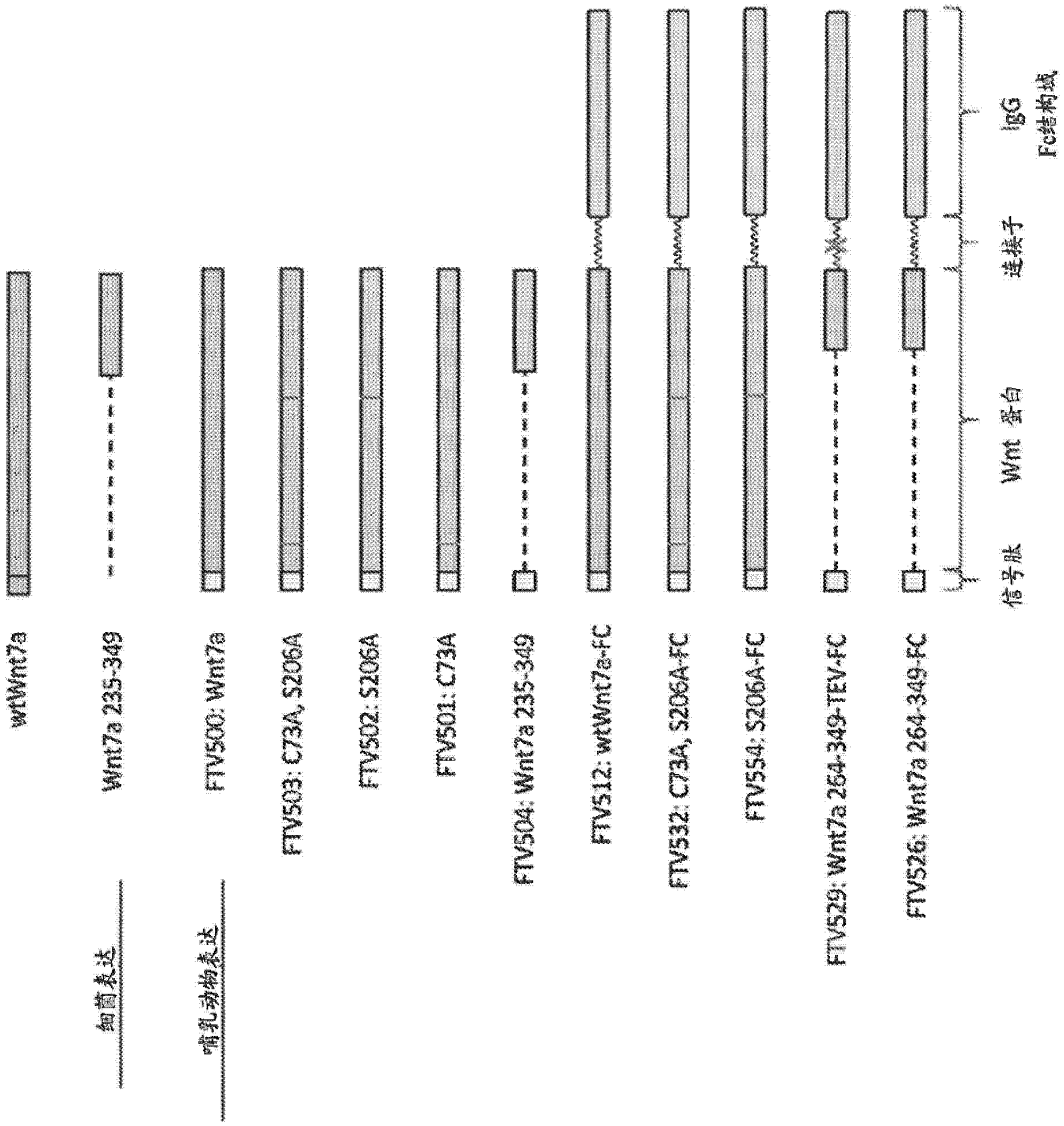


图 3

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WNT7A	融合物	表达 mg/L
野生型		0.9
野生型	Fc	4.6
C73, S206A		1.7
C73, S206A	Fc	11.9
C73A, S206A		1.4
C73A, S206A	Fc	12.0
aa264-349	Fc	10.8

图 4

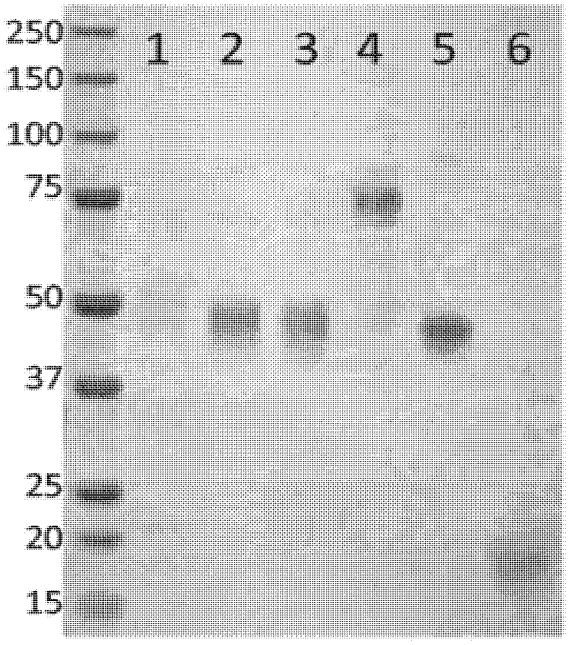


图 5

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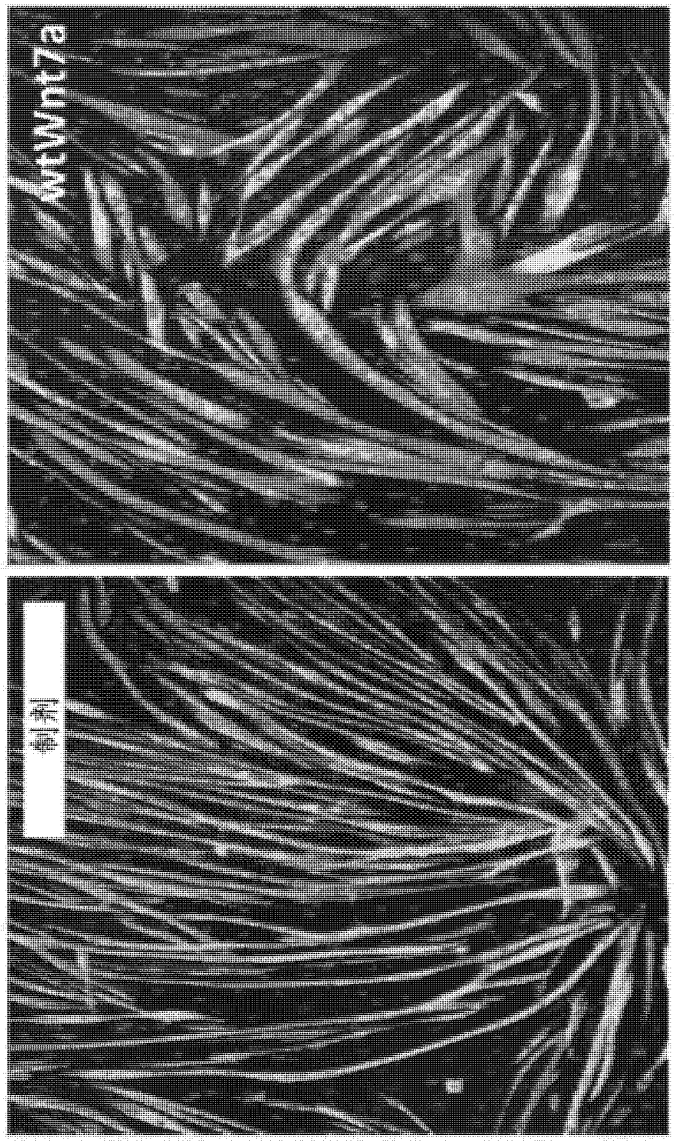


图 6

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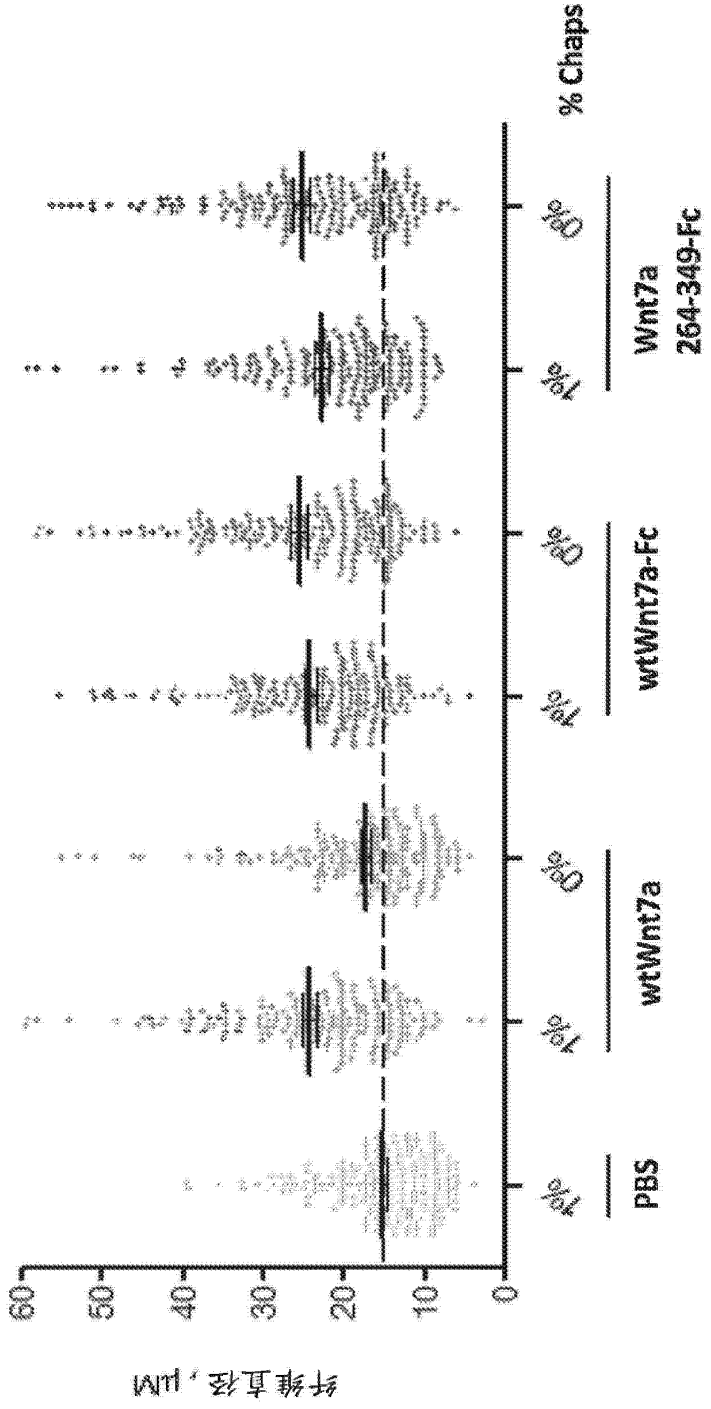


图 7

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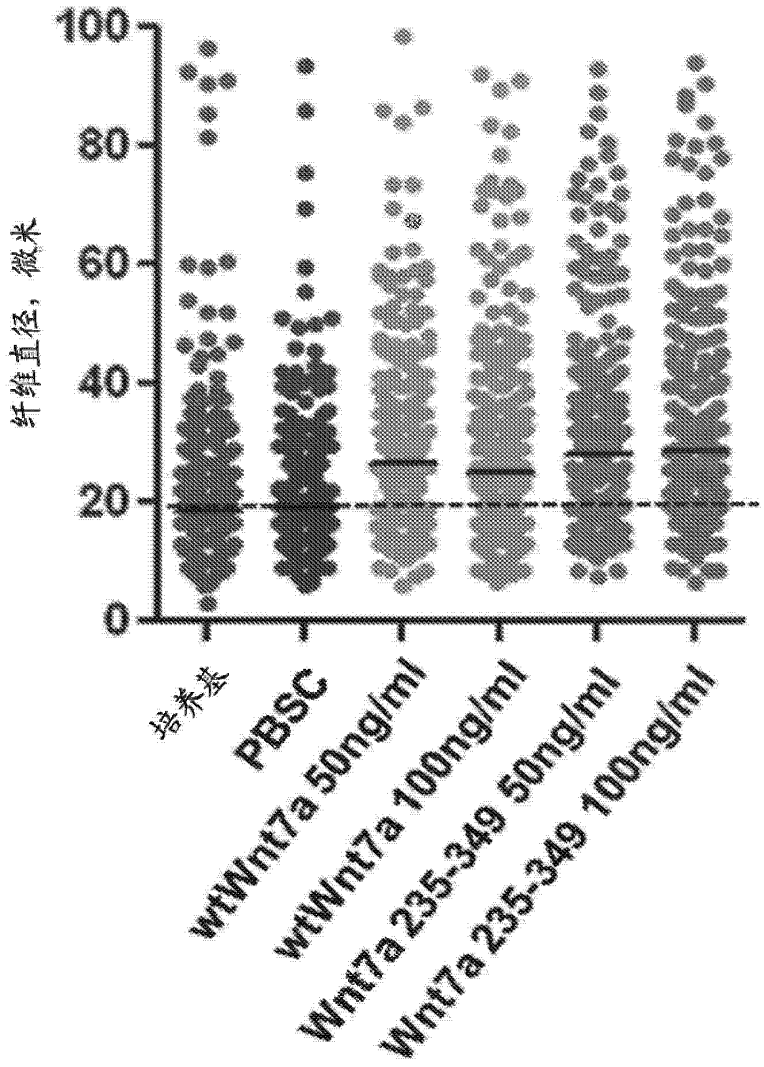


图 8A

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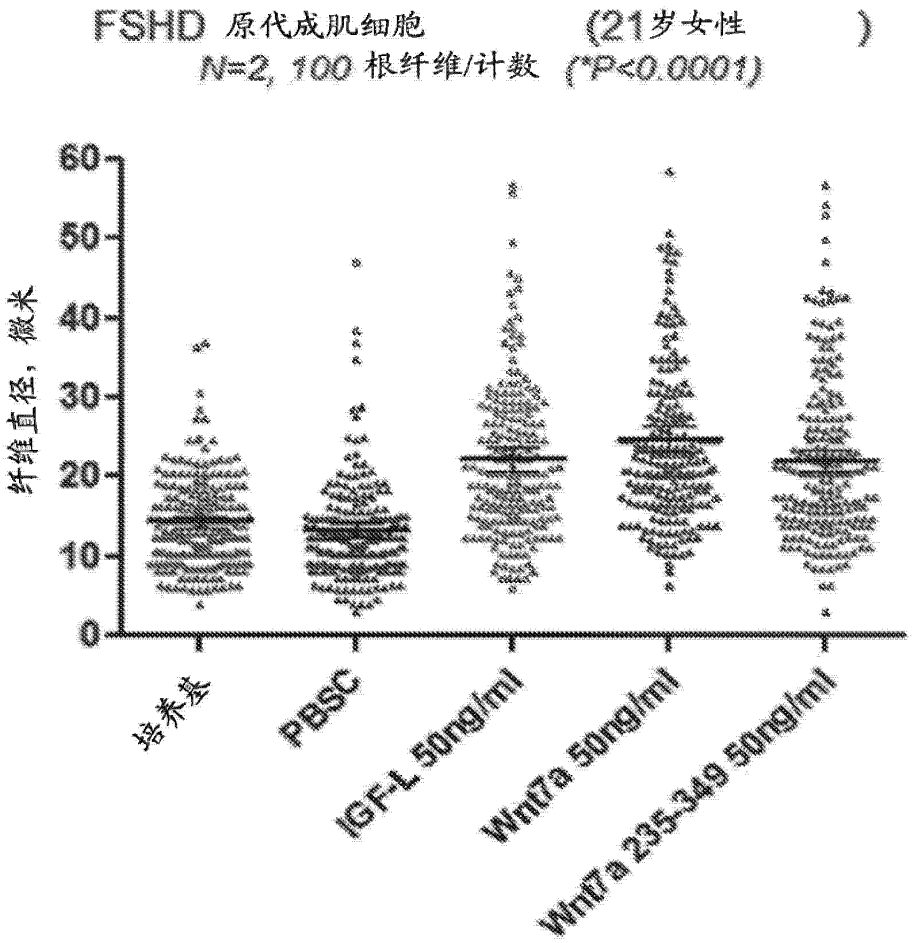


图 8B

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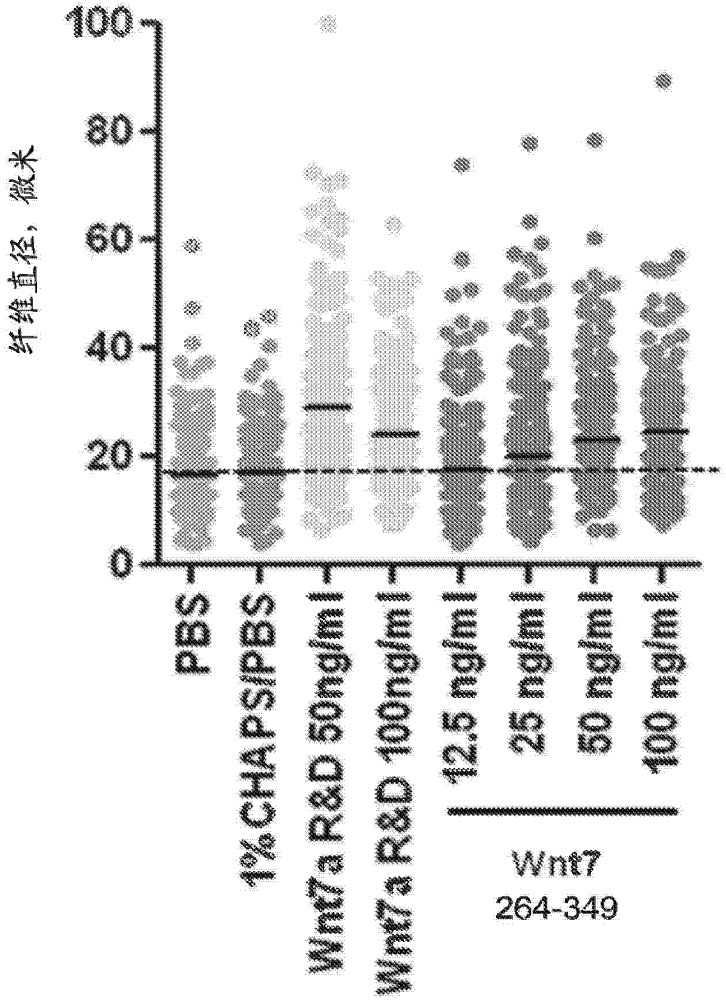


图 8C

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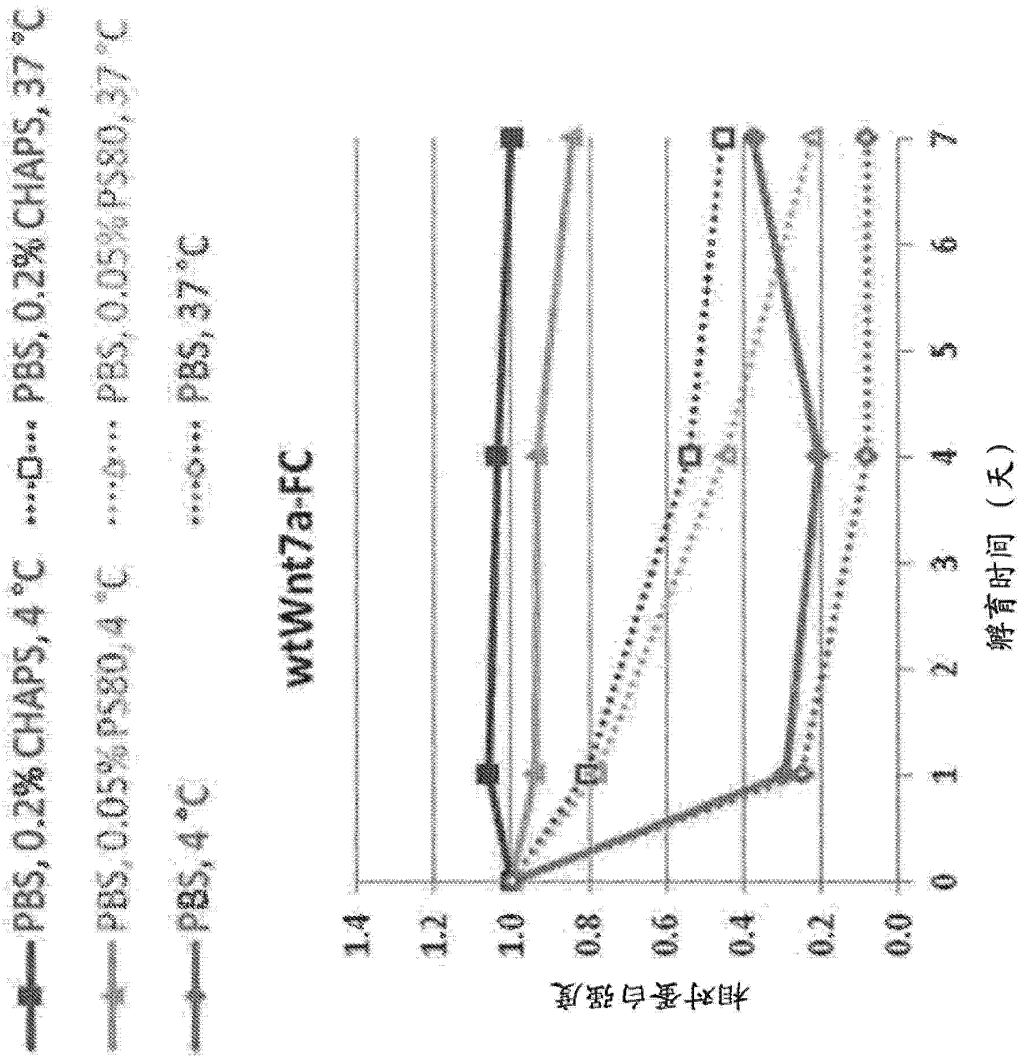


图 9A

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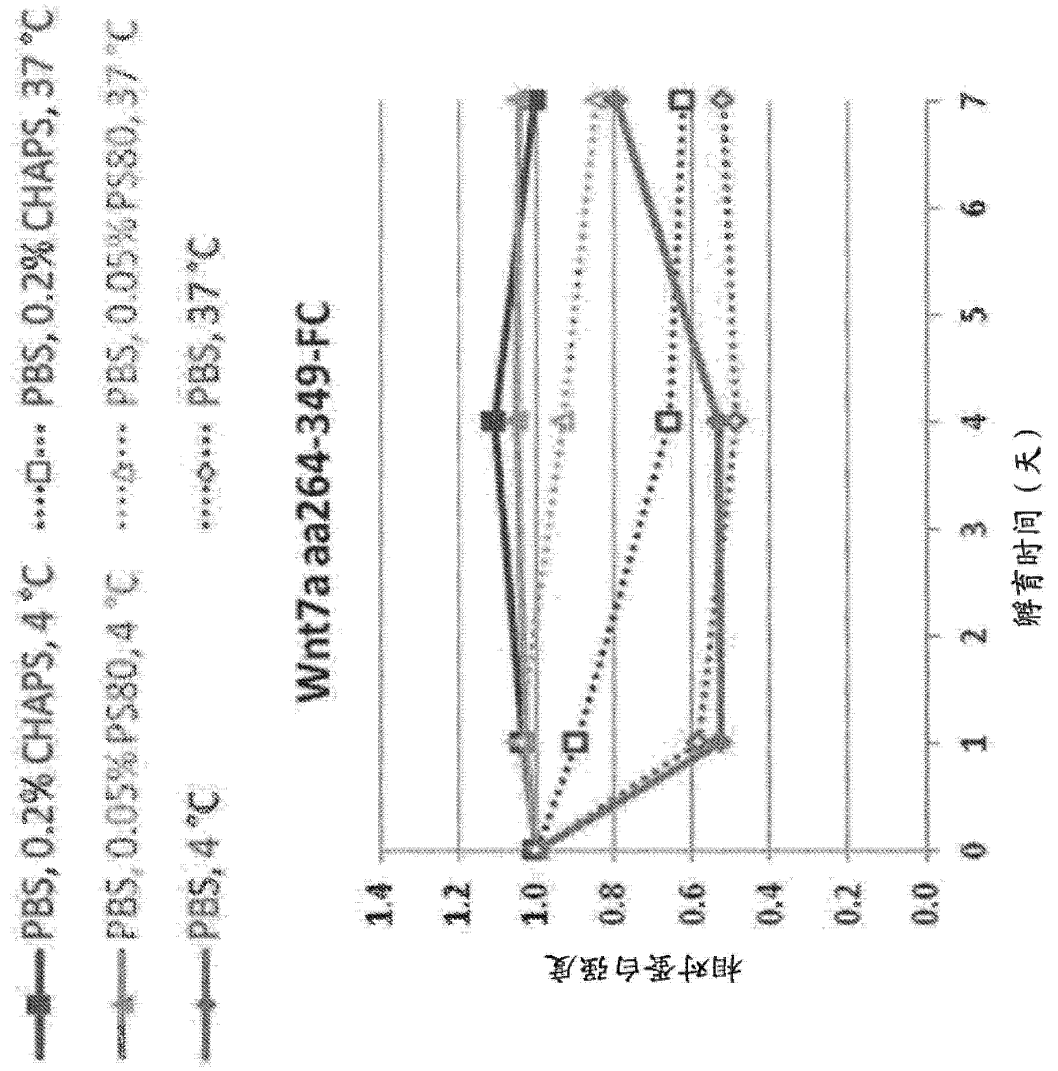


图 9B

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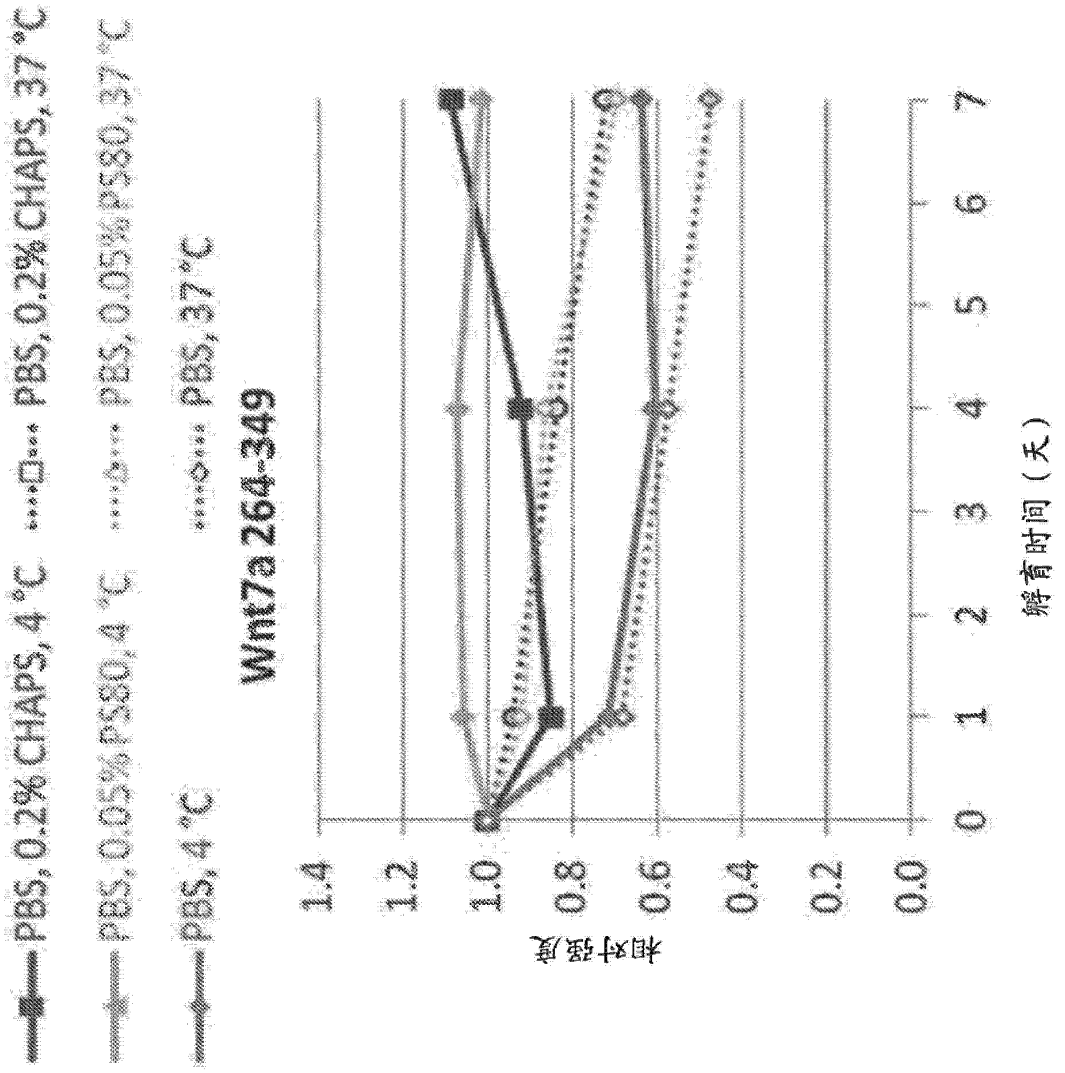


图 9C

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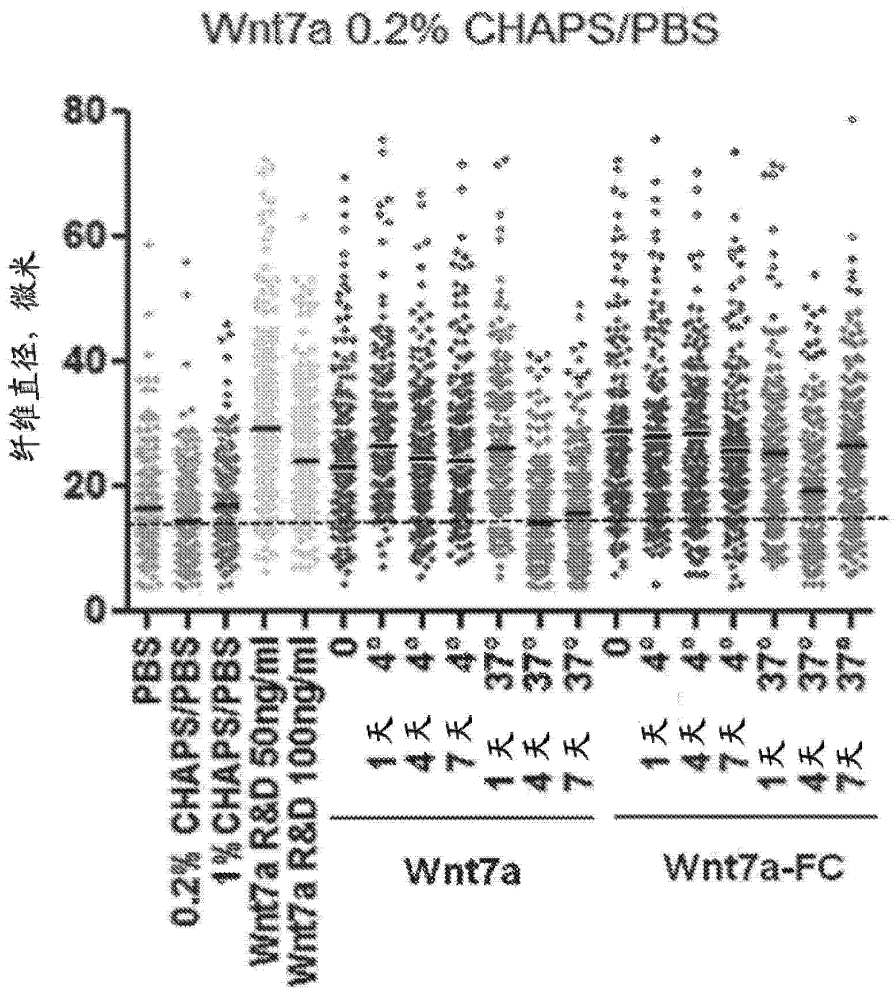


图 10A

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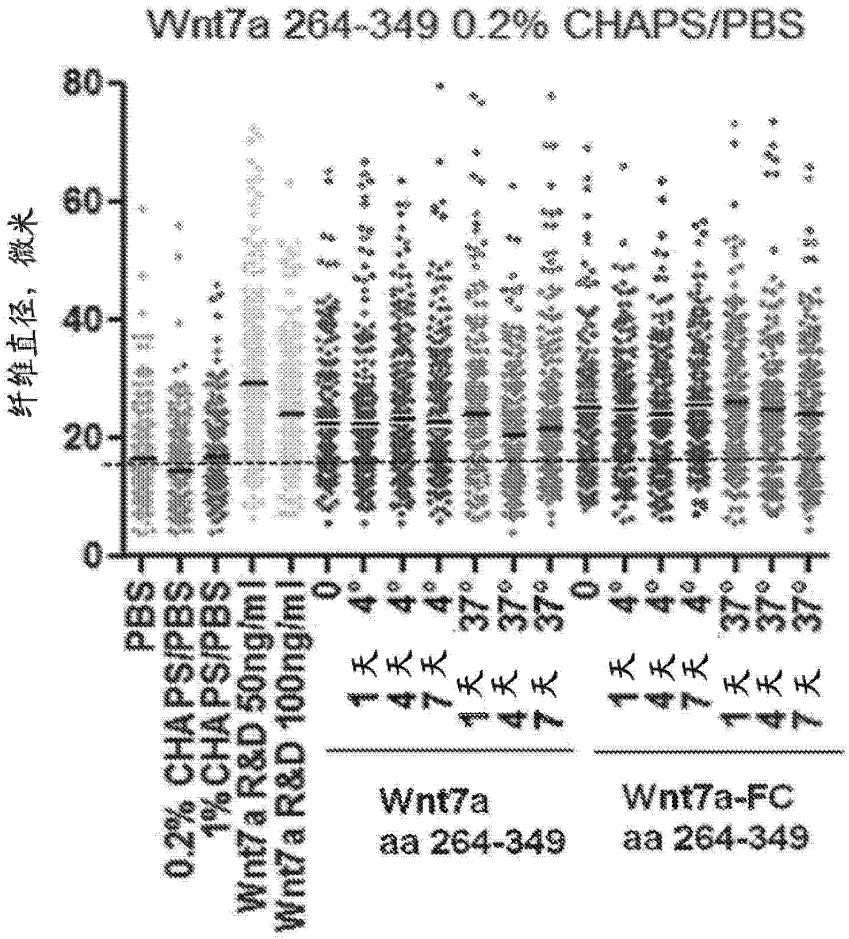


图 10B

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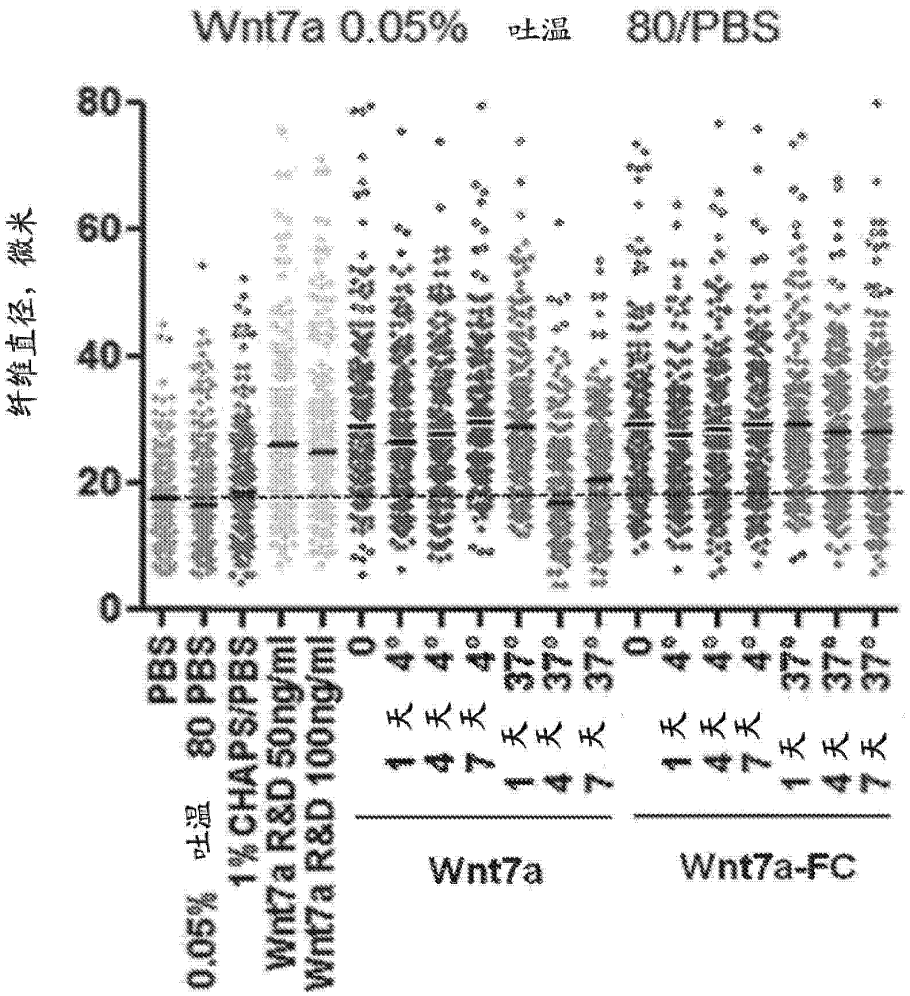


图 11A

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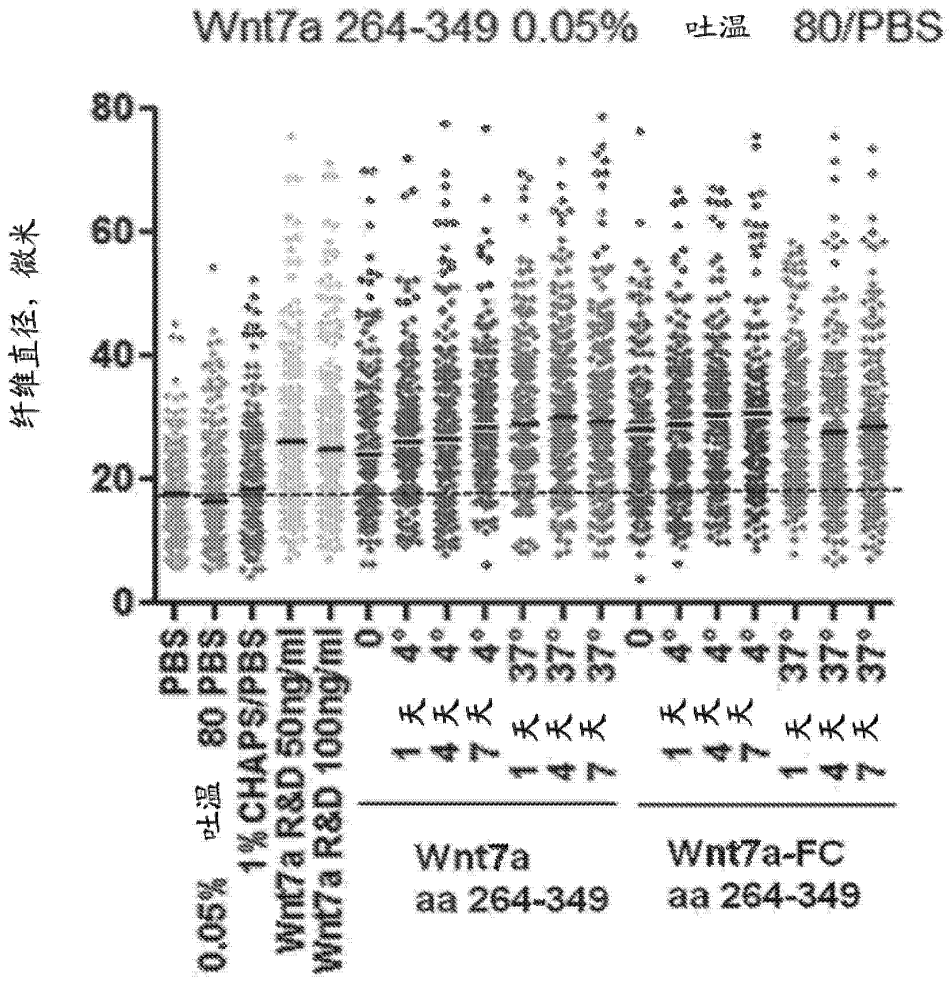


图 11B

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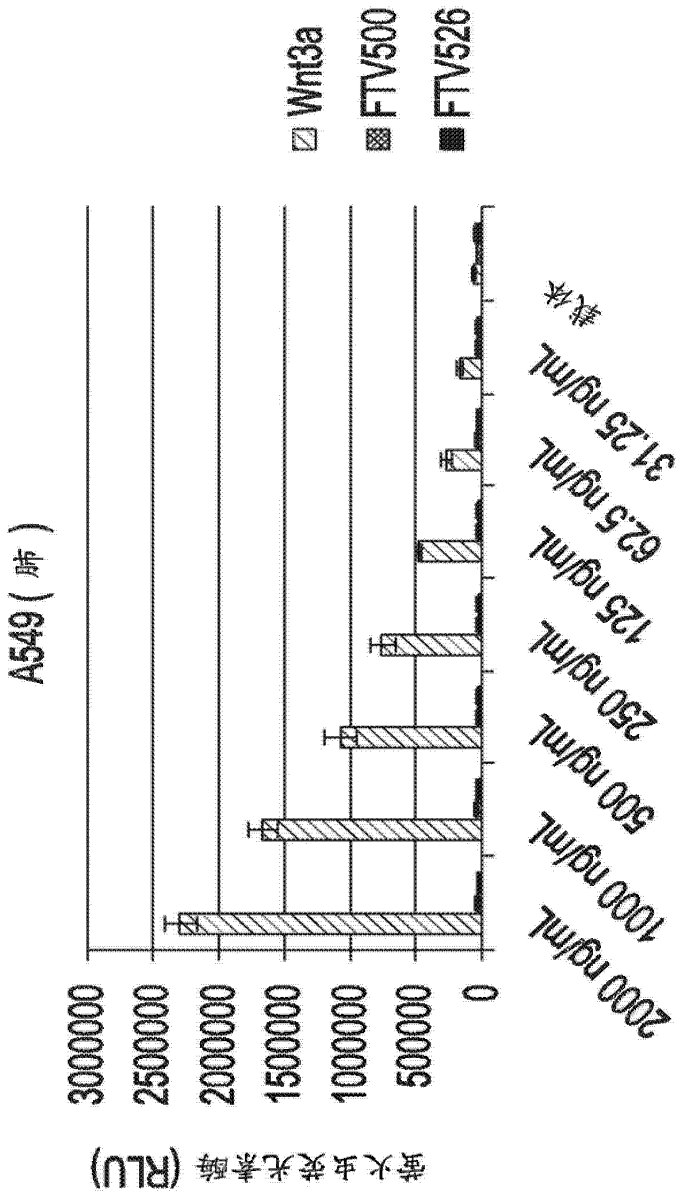


图 12A

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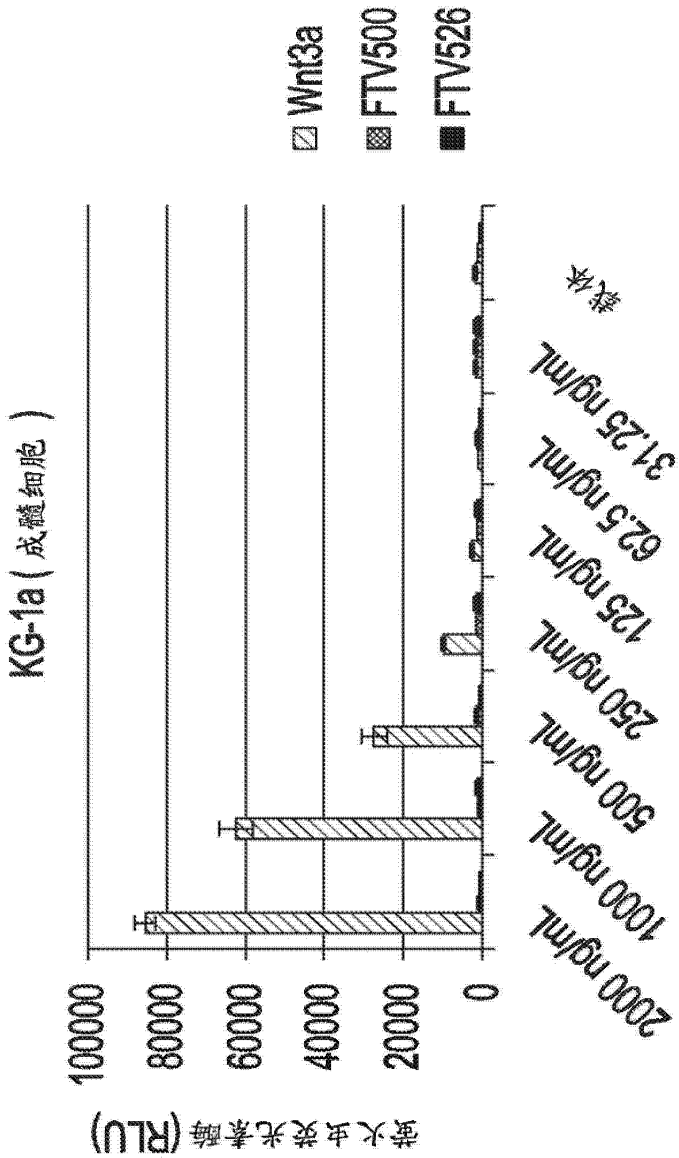


图 12B

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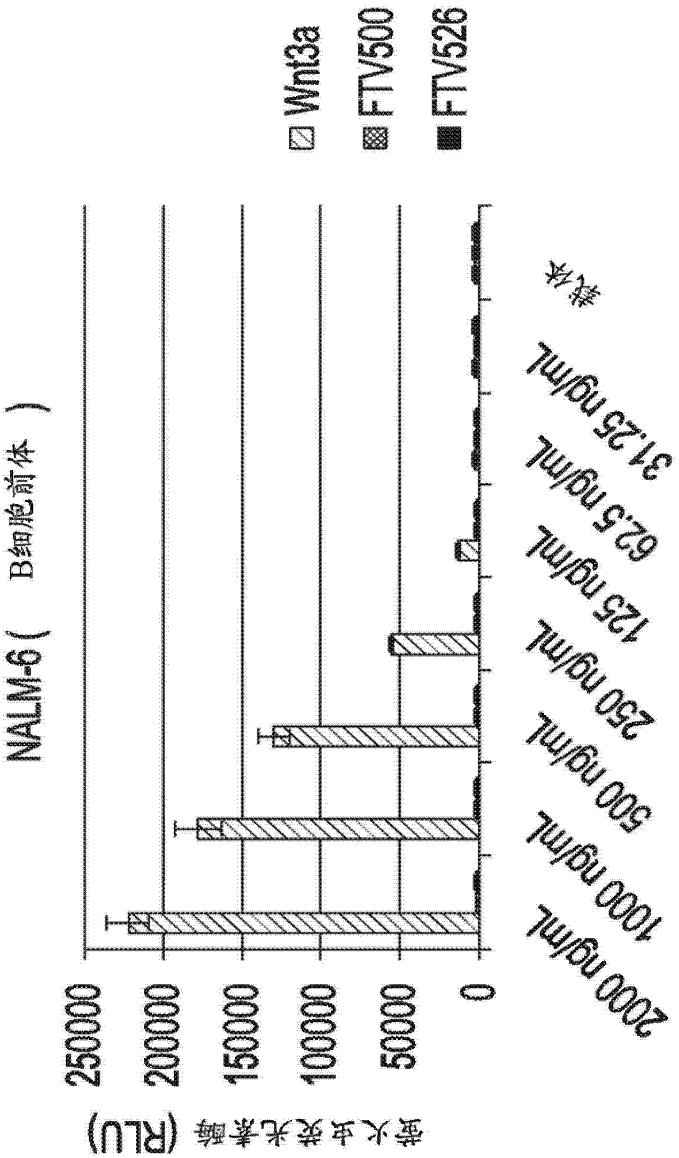


图 12C

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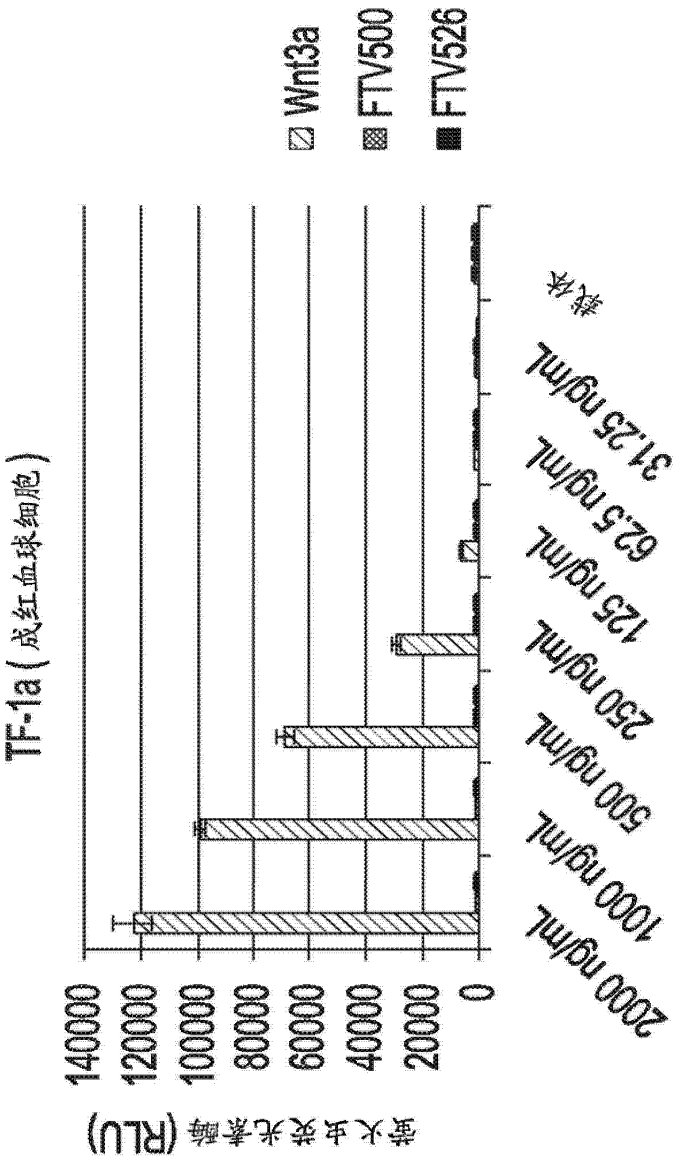


图 12D

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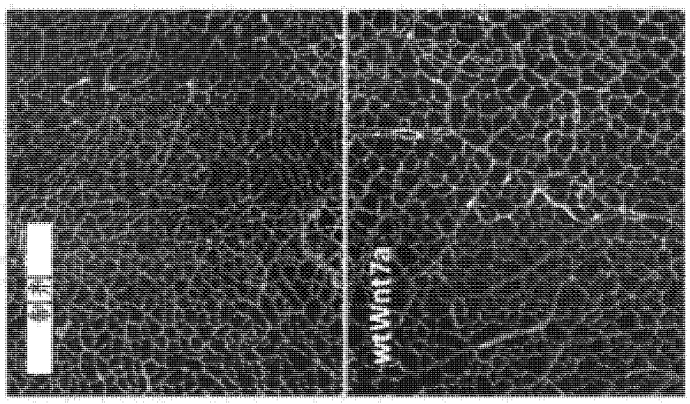


图 13A

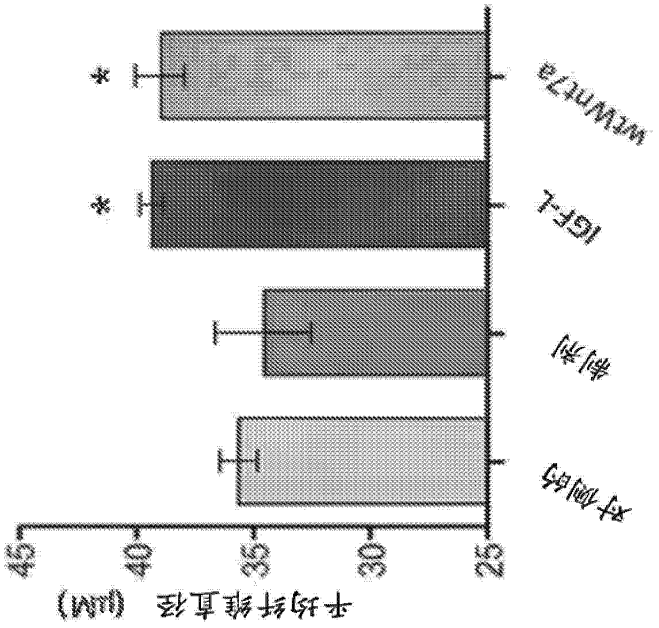


图 13B

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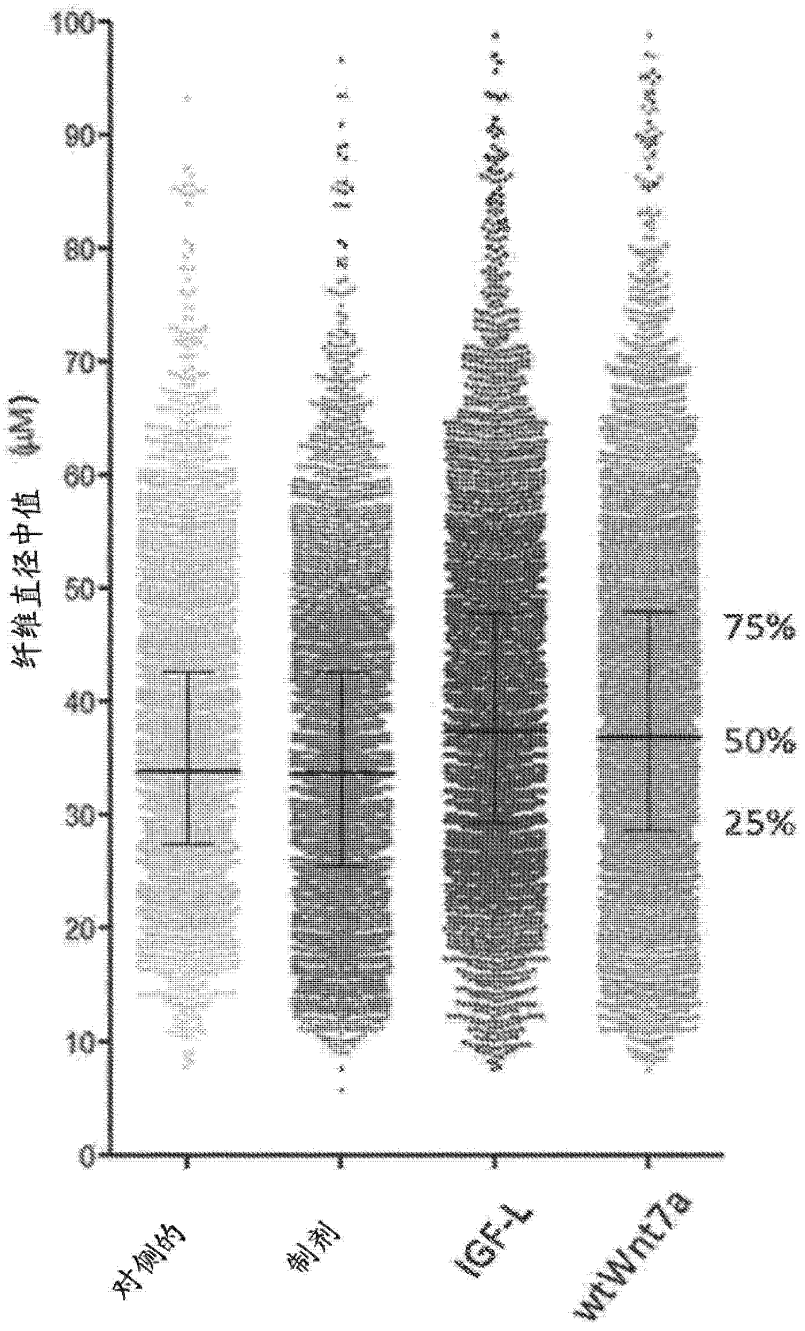
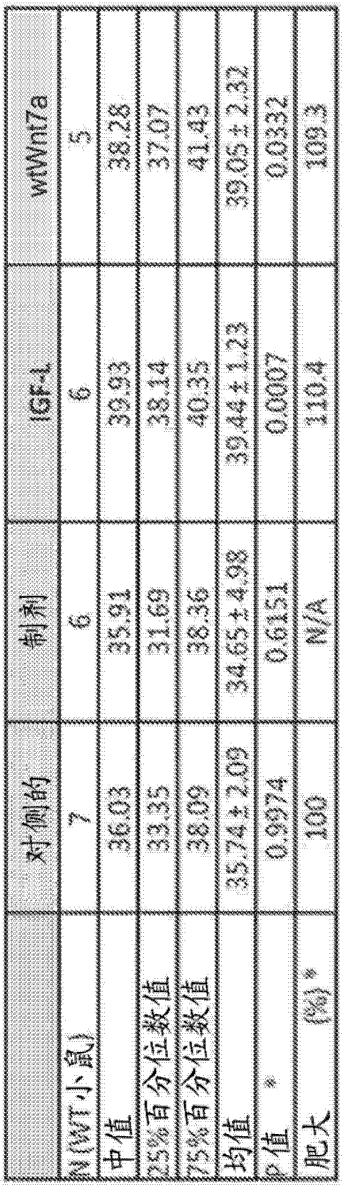


图 13C

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	对侧的	制剂	IGF-1	wtWnt7a
N(WT小鼠)	7	6	6	5
中值	36.03	35.91	39.93	38.28
25%百分位数值	33.35	31.69	38.14	37.07
75%百分位数值	38.09	38.36	40.35	41.43
均值	35.74 ± 2.09	34.65 ± 4.98	39.44 ± 1.23	39.05 ± 2.32
P值 *	0.9974	0.6151	0.0007	0.0332
肥大 (%) *	100	N/A	110.4	109.3

图 13D

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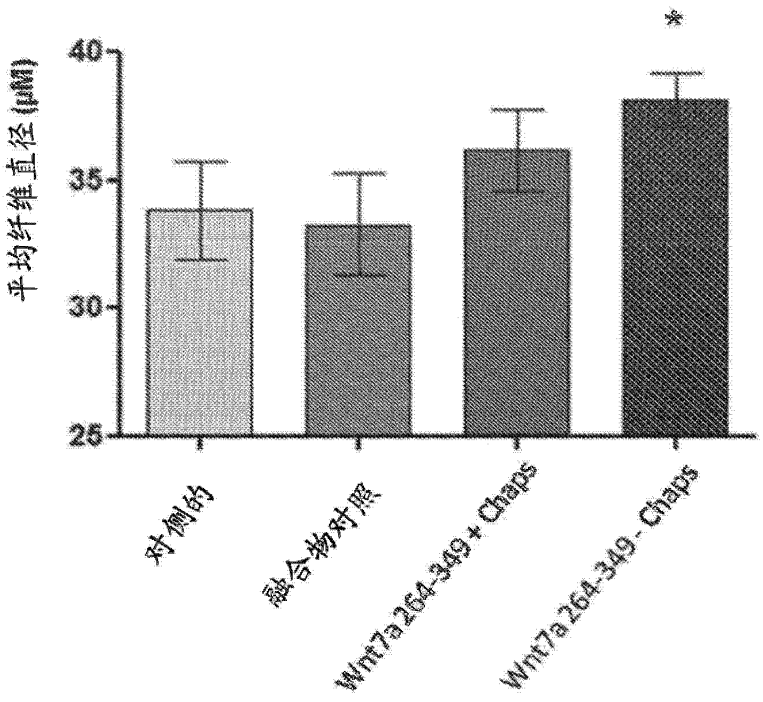


图 14A

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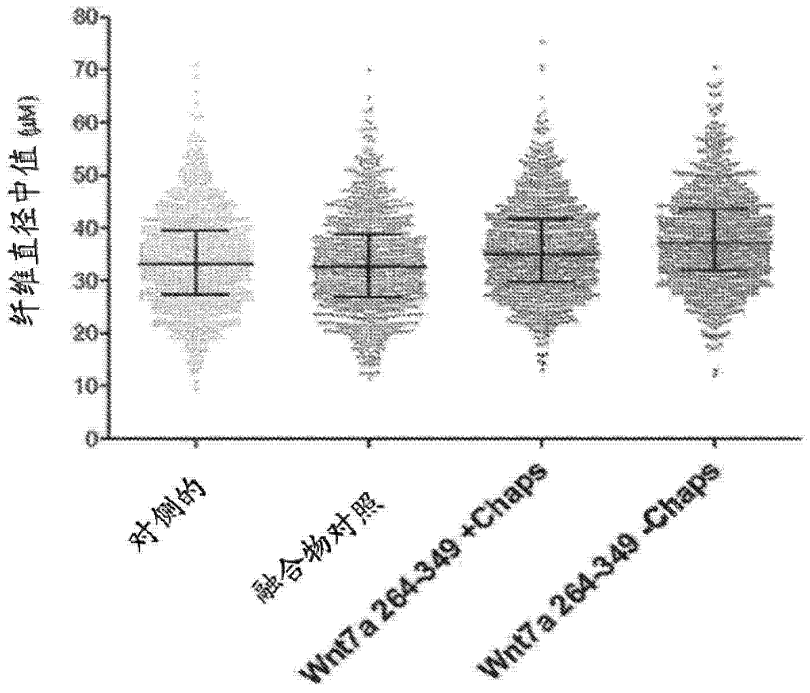
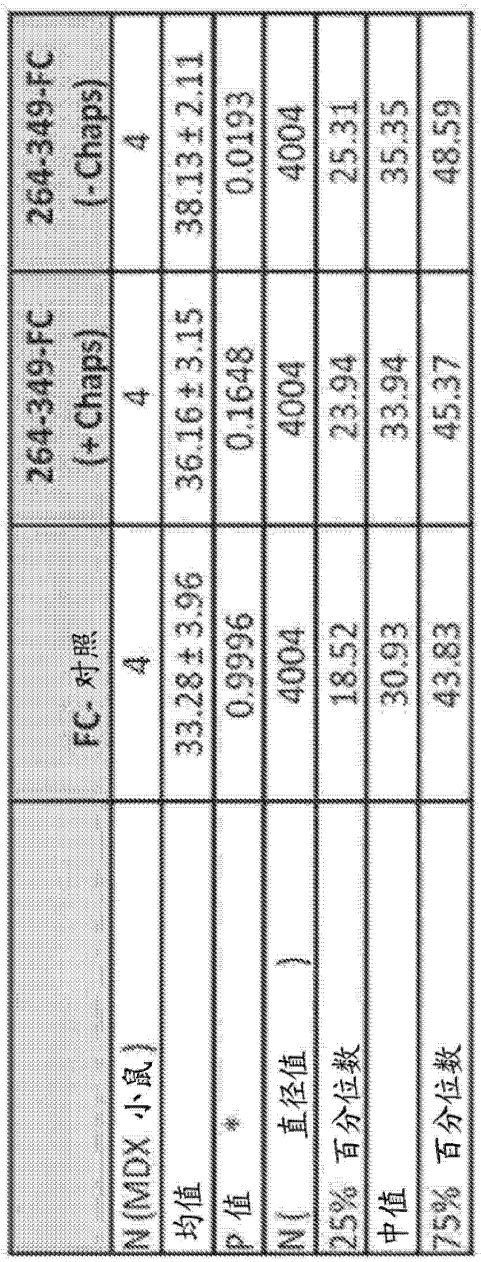


图 14B

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	FC- 对照	264-349-FC (+Chaps)	264-349-FC (-Chaps)
N (MDX 小鼠)	4	4	4
均值	33.28 ± 3.96	36.16 ± 3.15	38.13 ± 2.11
P 值 *	0.9996	0.1648	0.0193
N (直径值)	4004	4004	4004
25% 百分位数	18.52	23.94	25.31
中值	30.93	33.94	35.35
75% 百分位数	43.83	45.37	48.59

图 14C

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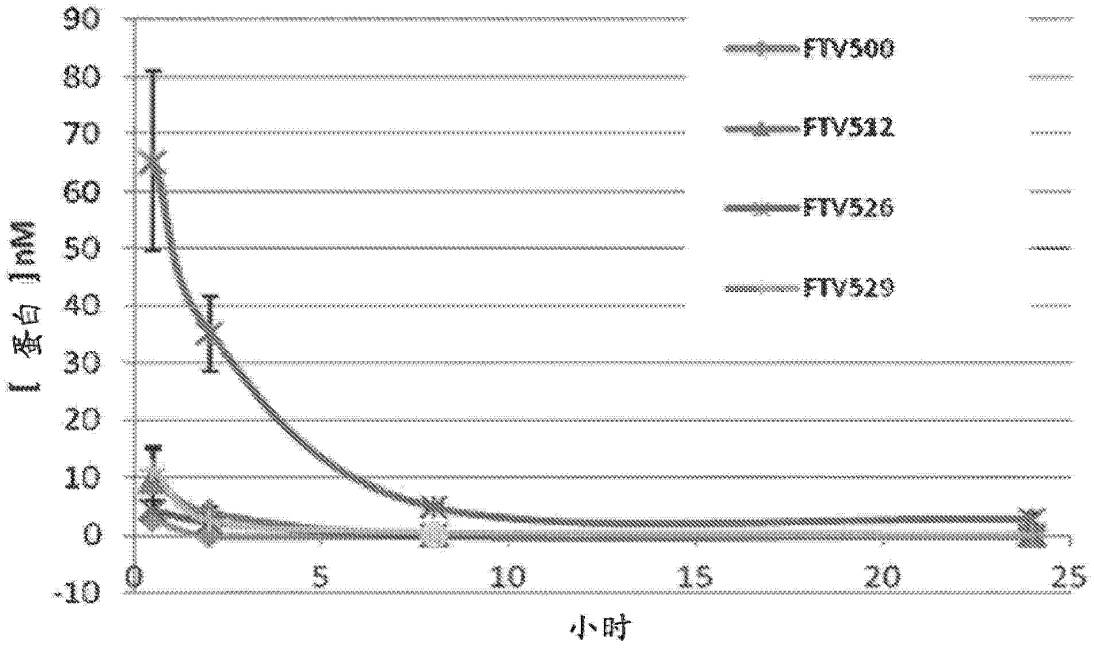


图 15A

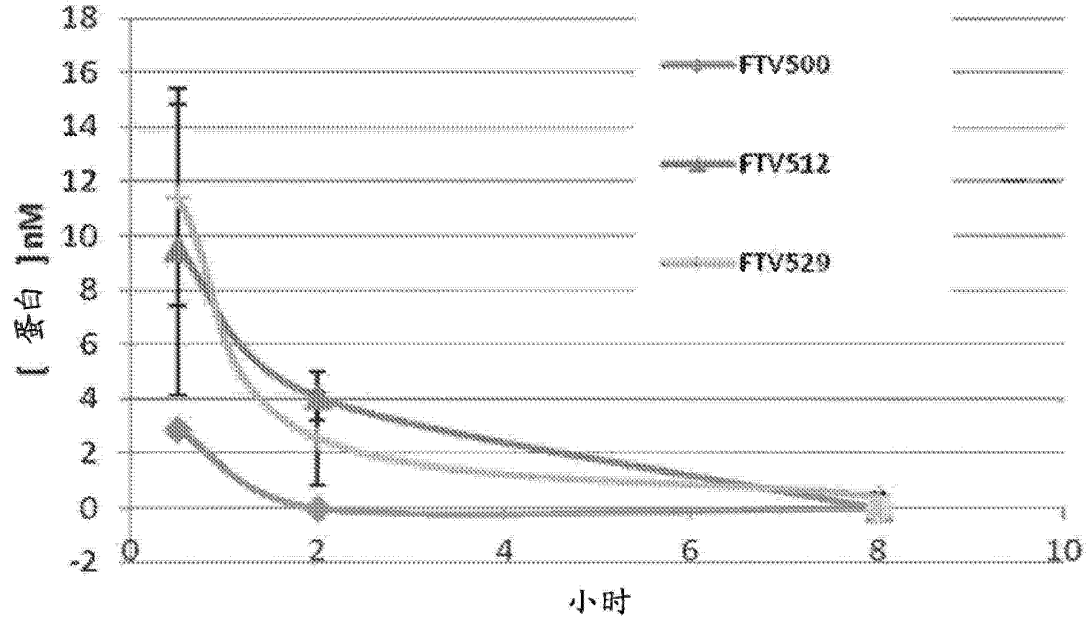


图 15B

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61/517,191 14 April 2011 (14.04.2011) US

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(54) Title: COMPOUNDS

(57) Abstract: The present invention relates to compounds that act as agonists of the Wnt signalling pathway, compositions comprising these compounds and the uses of these compounds, both therapeutic and in research. The invention also provides methods of identifying compounds that act as agonists of the Wnt signalling pathway.

WO 2012/140274 A2

COMPOUNDS

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

The present invention relates to compounds that act as agonists of the Wnt signalling pathway, compositions comprising these compounds and the uses of these compounds, both therapeutic and in research. The invention also provides methods of identifying compounds that act as agonists of the Wnt signalling pathway.

BACKGROUND

The Wnt signalling pathway plays key roles in diverse biological processes from development to tissue self-renewal and cancer. Wnt signals ultimately activate transcriptional programs that are known to be important in the maintenance and activation of stem cells, in promoting cellular proliferation, and controlling tissue expansion and cell fate determination[1]. Loss of components of the Wnt pathway can produce dramatic phenotypes that affect a wide variety of organs and tissues including, but not limited to the gut, hair follicles, hematopoietic system, and bone.

The canonical Wnt/β-catenin signalling pathway is characterised by a series of events that occur when a Wnt protein binds to a cell-surface receptor of a Frizzled (Frz) receptor family member in complex with an LRP co-receptor family member. This results in the activation of Dishevelled family proteins which inhibit a complex of proteins that includes axin, GSK-3, and the protein APC to degrade intracellular β-catenin. The resulting enriched nuclear β-catenin enhances transcription by TCF/LEF family transcription factors of a wide range of target genes including Lgr5, transcription of which is up-regulated by the Wnt pathway in colon cancer[2,3].

Rspondin (roof plate-specific spondin) proteins are also known to activate β-catenin signalling[4] and to drive proliferation of cells in the gut[5]. However, Rspondin proteins do

not bear sequence similarity to Wnt proteins, are not thought to bind to Frz receptors, and their receptor and mechanism for activating β-catenin signalling is unknown.

Alterations in Wnt and components of the Wnt signalling pathway are known to be associated with carcinogenesis. In view of the key role played by the Wnt signalling pathway in cancer, attempts have been made to develop antagonists of the pathway that may be useful in the treatment of cancer.

As the role of the Wnt signalling in cell growth and proliferation is elucidated, there is also significant interest in developing agonists that enhance the Wnt pathway for treating diseased or damaged tissue, for tissue regeneration and cell growth and proliferation, and tissue engineering.

Wnt/β-catenin signaling is known to increase proliferation of cells in the intestinal crypts[6]

In addition, Wnt/β-catenin signaling is known to increase bone mass by increasing the number of osteoblasts, cells which play a critical role in bone formation. Thus, it has been suggested that enhancing Wnt/β-catenin signaling to increase the number of osteoblasts, has the potential to provide novel therapeutic approaches for osteoporosis and related bone diseases[7].

Recently, it was demonstrated that agonising Wnt signalling, could protect against intestinal stem cell damage during graft-versus-host disease, a major complication during bone-marrow transplantation[8].

Wnt/β-catenin signalling has also been shown to promote hair follicle growth[9] by promoting the proliferation of matrix cells[10]. This suggests that the use of Wnt agonists may also be useful for treatment of baldness or prevention of hairloss during chemotherapy.

Wnt agonists also form a key component of stem cell culture media[11]. Therefore, controlled modulation of the Wnt/β-catenin signalling pathway could play an important role in improving culture media for cell growth and tissue engineering.

These are just a few examples of the many functions of the Wnt/β-catenin signalling pathway.

Known agonists of the Wnt/β-catenin signalling pathway include the Rspondin proteins (for example NU-206, Kirin Brewery Co Ltd), and small-molecule inhibitors of GSK-3 such as Stemolecule™ BIO (Stemgent®) and those shown in Table 4 of Rey and Ellies Developmental Dynamics 2010(239) 102-114, Norrin (also called Norrie Disease Protein, or NDP) which is a secreted regulator protein that binds with high affinity to Frizzled-4 and induces activation of the canonical Wnt signaling pathway[12], as well as soluble Wnt proteins and small molecules that mimic Wnt proteins (such as SC-222416).

A problem associated with these known agonists is that they can activate virtually all cells since, for example, GSK3 is ubiquitously distributed. A further disadvantage of using wild-type Rspondin as an agonist, may be that it contains regulatory sequences which could cause it to be activated or inactivated, making dose response predictions very difficult. It is thus difficult to direct agonistic activity to specific cell types and/or tissues and side effects are expected to be widely distributed. There is a need for compounds that can trigger activation of the canonical Wnt pathway in a more selected way without undesired side effects. There is also a need for compounds that can trigger activation of the canonical Wnt pathway in a more predictable manner for safe and predicable dosing regimes.

There is clearly a need to identify and develop further agonists of Wnt/β-catenin signalling for therapeutic reasons, as well as for tissue engineering and cell culture.

SUMMARY OF INVENTION

Accordingly, the invention provides an agonist of the Wnt pathway which mimics the activity of an Rspondin protein binding to an Lgr protein.

Surprisingly, the inventors have found that the Lgr protein family members, Lgr4, Lgr5 and Lgr6, are facultative Wnt receptor components that mediate Rspondin signaling. In particular, the inventors have established that the Lgr proteins form a complex with Frizzled receptors and LRP proteins to which both Wnt and Rspondin bind. Binding of Rspondin to the Lgr proteins in the complex promotes Wnt/β-catenin signalling in the presence of Wnt. The inventors have shown that Lgr proteins can occur in a physical complex with Frizzled receptors and LRP proteins and bind soluble Rspondins. Engagement of Lgr proteins (Lgr4 or Lgr5 or Lgr6) by Rspondin proteins triggers downstream canonical Wnt signals through the associated Frizzled-LRP complex in the presence of Wnt. It is postulated that binding of Wnt to Frizzled receptors and LRP proteins within the complex induces conformational or biochemical changes in the receptor complex that are essential for the subsequent enhancement of signalling activity by Rspondin/Lgr interaction.

The mechanism for Rspondin-mediated enhanced Wnt/β-catenin signalling identified by the inventors is surprising for a number of reasons. Firstly, the interaction between Lgr proteins and Frizzled receptors and LRP proteins has not been previously described. Additionally, it is unexpected that Lgr, one of the Wnt target genes, should itself participate in Wnt signaling. It is also surprising that the Lgr-Frz-LRP receptor complex (termed herein the Wnt receptor complex) has two ligands, a feature only rarely found in nature.

Another surprising feature is that Lgr5 rarely occurs alone but is usually expressed in cells also expressing Lgr4. In contrast to this, both Lgr4 and Lgr6 are expressed exclusively in certain cells and tissues. This knowledge may be used to design compounds that enhance Wnt signaling in particular cells or tissues. For example, an Lgr6 agonist might specifically bind to epidermal cells, for which Lgr6 is an exclusive marker, and enhance Wnt signaling in these compartments e.g. to treat epithelial

damage. By contrast, an Rspondin molecule, used to treat the same epithelial damage, would target all cells expressing Lgr4, Lgr5 or Lgr6 proteins and would be likely to cause a number of unwanted side-effects as a result. Conversely, these expression patterns also mean that treatments focusing solely on Lgr5 inhibition are likely to be redundant due to the presence and overlapping functionality of Lgr4 and/or Lgr6.

There are four secreted Rspondin proteins (Rspo1, Rspo2, Rspo3 and Rspo4), only encoded in vertebrate genomes. Loss-of-function mutations in Rspo1, Rspo 3 and Rspo 4 result in similar phenotypic defects to those seen in patients or mice lacking expression of Wnt ligands and/or receptors[13, 14, 15]. Rspondin proteins do not bear sequence similarity to Wnt family proteins, but are often co-expressed with and induced by Wnt genes during embryogenesis. Rspondin proteins are particularly potent β-catenin activators when also present in the presence of secreted Wnt proteins.

Several theories have been suggested as to how Rspondin-1 might promote Wnt/β-catenin signalling: firstly, Rspondin-1 has been reported to be a high-affinity ligand of the Wnt co-receptor LRP6[16]; secondly, Rspo1 has been postulated to bind and block the Kremen protein that down-regulates surface expression of Wnt receptors[17]; thirdly, Rspo1 has been proposed to activate the Wnt pathway by blocking the interaction of the Wnt inhibitor Dickkopf1 (DKK1) with the LRP6 coreceptor[18], and finally, identification of an interaction between Rspo1 and adult stem cell marker Lgr5 has led to the proposal of an *alternative pathway* for the activation of β-catenin signalling[19]

However, despite these studies, the exact nature of the Rspondin receptors and the mechanism by which Rspondin drives canonical Wnt/β-catenin signals was unknown prior to the work by the inventors described herein. The work conducted by the present inventors provides the first realisation that the interaction between Rspondin and Lgr is part of a more complicated signalling mechanism requiring the presence of Frizzled Receptors and LRP proteins. The inventors are also the first to demonstrate that Rspondin proteins not only bind to Lgr4, Lgr5 and Lgr6 proteins but moreover they demonstrate the requirement of Lgr proteins for Rspondin-mediated activation of Wnt/β-catenin signalling, and thus show that Lgr proteins act as receptors for Rspondin that, in

complex with Wnt, Frizzleds and LRPs, mediate enhanced canonical Wnt/β-catenin signalling. The authors have also demonstrated for the first time, that through binding to Lgr5, which is only expressed in stem cells, Rspondin plays a role in the activation and growth or stem cells.

Although blocking of binding of Rspondin proteins to Lgr5 by anti-Lgr5 antibodies was previously demonstrated and an *alternative pathway* for the activation of β-catenin signalling was hypothesised in WO2009/005809, the authors of this publication did not show effects on signalling by Rspondin-blocking Lgr5 antibodies, nor did they show any data of anti-Lgr5 antibodies in *in vivo* cancer models. No model for how Rspondin might mediate β-catenin signaling was proposed. Furthermore, given the latest data provided in the present invention showing that Lgr5 is only ever expressed in cells also expressing Lgr4, it is highly unlikely, that even if the authors of WO2009/005809 had tested their anti-Lgr5 antibody in *in vivo* cancer models, that they would have worked (i.e. treated the cancer and reduced the size of the tumour). This is because, although the antibody may have blocked Lgr5-mediated Wnt enhancement, Lgr4-mediated Wnt enhancement would be unaffected so the levels of Wnt activation would probably have remained at a similar level. The authors would not have known this at the time. Armed with the new knowledge about the Lgr4/Lgr5 expression patterns, as provided in the present application, it would be possible for a person skilled in the art to develop antagonists that block both Lgr4 and Lgr5 and thus much more effectively block Rspondin-mediated Wnt enhancement and provide a far more effective treatment to cancer and other diseases that are known to be treated by inhibition of Wnt signalling.

Furthermore, WO2009/005809 does not make the link between Lgr5 and stem cells. Therefore, the authors could not have known that Lgr5 was involved in normal stem cell growth. As such there is no suggestion to design agonists of Lgr5 (or of Lgr4 or Lgr6) as described herein. The skilled person would not be motivated by WO2009/005809 to design agonists of Lgr5 for stimulating stem cell growth.

The present inventors have also discovered that Lgr proteins are located on the basolateral side of epithelial cells. This knowledge makes possible the targeting of

Rponding-mimicking agonists to the basolateral side of epithelial cells using other targets, for example tissue-specific targets, that are also known to be expressed basolaterally.

The identification of this surprising mechanism for Rspondin-induced Wnt/β-catenin signalling through the Lgr-Frizzled-LRP complex presents new opportunities for designing Rspondin mimics to activate Wnt/β-catenin signalling.

Rspondin proteins

The Rspondin protein that is mimicked by the agonist of the invention may be any known Rspondin protein since all of these Rspondin proteins are expected to fulfil the same role in the Wnt signalling pathway.

The Rspondin protein may thus be Rspondin 1 (gi: 284654; SEQ ID NO: 112), Rspondin 2 (gi: 340419; SEQ ID NO: 113); Rspondin 3 (gi: 84870; SEQ ID NO: 114) or Rspondin 4 (gi: 343637; SEQ ID NO: 115).

Lgr proteins

Lgr proteins are markers of adult stem cells[20]. The agonist of the invention may mimic the effect of the Rspondin protein binding to any one of the Lgr family of proteins. In particular, the agonist of the invention may mimic the effect of the Rspondin protein binding to Lgr4 (gi: 55366), Lgr5 (gi: 8549) or Lgr6 (gi: 59352). Lgr4, Lgr5 and Lgr6 are all seven-transmembrane receptors that are close relatives of the receptors for the hormones FSH, LH and TSH.

Lgr5 is a Wnt target gene whose expression marks proliferative stem cells in multiple Wnt-dependent stem cell compartments, such as the small intestine and colon, the stomach, pancreas, liver, kidney, middle-ear, mammary gland, and the hair follicle. An agonist of the invention that mimics the effect of an Rspondin protein binding to Lgr5 would thus enhance canonical Wnt signals in all $Lgr5^{+}$ cells, and hence increase proliferation in the stem cell compartment.

Lgr4 is much more widely expressed than Lgr5[21]. The inventors have previously shown that Lgr5 is co-expressed with Lgr4 in the stem-cell compartments mentioned above. Thus, Lgr5 marks intestinal stem cells at the base of crypts, while Lgr4 marks all crypt cells, including the $Lgr5^+$ stem cells. An agonist of the invention that mimics the effect of an Rspondin protein binding to Lgr4 would enhance canonical Wnt signals in all $Lgr4^+$ cells. Lgr4 is more widely expressed than Lgr5 and therefore, an agonist mimicking the effect of an Rspondin protein binding to Lgr4 of the invention would be expected to enhance Wnt signals and proliferation in a wider set of cells than a compound targeting Lgr5. For example, in the small intestine, all crypt cells express Lgr4 and therefore, an agonist of the invention that mimics the effect of an Rspondin protein binding to Lgr4 would enhance proliferation of all crypt cells.

Lgr6 marks multipotent stem cells in the epidermis[22] and Lgr6 positive cells play a crucial role in wound healing and generation of smooth muscle tissues in many organs (e.g. airways, large arteries, uterus). An agonist of the invention that mimics the effect of an Rspondin protein binding to Lgr6 would enhance canonical Wnt signals in all $Lgr6^+$ cells, and hence increase proliferation of these multipotent stem cells.

The inventors have discovered that the Lgr proteins are expressed on the basolateral side of epithelial cells. The basolateral membrane of a polarized cell is the surface of the plasma membrane that forms its basal and lateral surfaces. It faces towards the interstitium, and away from the lumen.

Wnt receptor complex

As discussed above, the inventors have shown that Rspondin proteins facilitate Wnt-induced activation of the canonical Wnt pathway by binding to the Lgr protein when it is in a complex with one or more Frizzled (Frz) receptors and LRP proteins and optionally, other Lgr proteins. The agonist of the invention may therefore mimic the effect of the Rspondin protein binding to at least one Lgr protein in a complex with at least one Frz receptor and at least one LRP co-receptor. It was observed, for example, that a Wnt receptor complex can comprise at least two Lgr proteins (see fig. 3). The Frz receptor in the complex is at least one of Frizzled 1 (Frz1; gi: 8321), Frizzled 2 (Frz2; gi: 2535),

Frizzled 3 (Frz3; gi: 7976), Frizzled 4 (Frz4; gi: 8322), Frizzled 5 (Frz5; gi: 7855), Frizzled 6 (Frz6; gi: 8323), Frizzled 7 (Frz7; gi: 8324), Frizzled 8 (Frz8; gi: 8325), Frizzled 9 (Frz9; gi: 8326), and Frizzled 10 (Frz10; gi: 11211). In one example, the complex may comprise at least one of Frz5, Frz6 and Frz7. The LRP co-receptor in the complex may be any LRP protein family member. The complex must thus comprise at least one of LRP5 (gi: 4041) and/or LRP6 (gi: 4040).

The agonist of the invention may therefore mimic the effect of an Rspondin protein binding to an Lgr protein, wherein the Lgr protein, which may be Lgr4, Lgr5 or Lgr6, is in a complex with, for example, Frz5-LRP6, Frz6-LRP6, Frz7-LRP6 and other combinations of the proteins described above. In particular, the agonist of the invention may mimic the effect of an Rspondin protein binding to at least one Lgr protein, wherein the Lgr protein is Lgr4, Lgr5 and/or Lgr6 and is in a complex comprising Frz5, Frz6 and/or Frz7, and LRP5 and/or LRP6.

These complexes may further comprise a Wnt protein, such as WNT1 (BC074799; gi: 7471), WNT2 (BC029854; gi: 7472), WNT2b/13 (BC141825; gi: 7482), WNT3 (BC112118; gi: 7473), WNT3a (BC103921; gi: 89780), WNT4 (BC057781; gi: 54361), WNT5a (BC064694; gi: 7474), WNT5B (BC001749, gi: 81029), WNT6 (BC004329; gi: 7475), WNT7a (BC008811; gi: 7476), WNT7b (BC034923; gi: 7477), WNT8a (NM_058244; gi: 7478), WNT8b (BC156632; gi: 7479), WNT9a (BC111960; gi: 7483), WNT9b (BC064534; gi: 7484), WNT10A (BC052234; gi: 80326), WNT10b (BC096353; gi: 7480), WNT11 (BC113388; gi: 7481), or WNT16 (BC104919; gi: 51384) (the first number in each bracket is a gene number as defined at: http://genome.ucsc.edu/cgi-bin/hgGateway). Preferentially, the complexes comprise Wnt5a or Wnt5B. The term "Wnt receptor complex" is used herein to refer to any such complex comprising at least one Lgr protein, an Frz receptor and an LRP protein, and optionally a Wnt protein.

Functional assays

The term "Rspondin-mimicking activity" refers to the ability of an agonist to mimic the effect or activity of an Rspondin protein binding to an Lgr protein. The ability of the agonists of the invention to mimic the activity of an Rspondin protein binding to an Lgr

protein can be confirmed by a number of assays. The agonists of the invention typically initiate a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand Rspondin. In particular, the agonists of the invention enhance the canonical Wnt/β-catenin signalling pathway. As used herein, the term "enhances" refers to a measurable increase in the level of Wnt/β-catenin signalling compared with the level in the absence of Rspondin or in the absence of an agonist of the invention that mimics Rspondin.

Various methods are known in the art for measuring the level of canonical Wnt/β-catenin signalling. These include, but are not limited to assays that measure: Wnt/β-catenin target gene expression; TCF reporter gene expression; beta-catenin stabilization; LRP phosphorylation; Axin translocation from cytoplasm to cell membrane and binding to LRP.

The canonical Wnt/β-catenin signalling pathway ultimately leads to changes in gene expression through the transcription factors TCF7, TCF7L1, TCF7L2 and LEF. The transcriptional response to Wnt activation has been characterised in a number of cells and tissues[23, 24, 25, 26, 27, 28, 29, 30]. As such, global transcriptional profiling by methods well known in the art can be used to assess Wnt/β-catenin signalling activation.

Changes in gene expression are generally mediated by TCF and LEF transcription factors. A TCF reporter assay assesses changes in the transcription of TCF/LEF controlled genes to determine the level of Wnt/β-catenin signalling. A TCF reporter assay was first described by Korinek, V. et al., 1997[31]. Also known as TOP/FOP this method involves the use of three copies of the optimal TCF motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal c-*Fos* promoter driving luciferase expression (pTOPFLASH and pFOPFLASH, respectively) to determine the transactivational activity of endogenous β-catenin/TCF4. A higher ratio of these two reporter activities (TOP/FOP) indicates higher β-catenin/TCF4 activity.

Various other reporter transgenes that respond to Wnt signals exist intact in animals and therefore, effectively reflect endogenous Wnt signalling (reviewed in Barolo, 2006[32]). These reporters are based on a multimerized TCF binding site, which drives expression

of LacZ or GFP, which are readily detectable by methods known in the art. These reporter genes include: TOP-GAL[33] BAT-GAL[34] ins-TOPEGFP, ins-TOPGAL[35], LEF-EGFP[36] Axin2-LacZ[37], Axin2-d2EGFP[38], Lgr5tm1(cre/ERT2)[39], TOPdGFP[40].

The recruitment of dephosphorylated β-catenin to the membrane[41], stabilisation and phosphorylation status of β-catenin[42] and translocation of β-catenin to the nucleus (Klapholz-Brown Z et al., PLoS One. 2(9) e945, 2007)[43] in some cases mediated by complex formation with TCF transcription factors and TNIK[23, 44] are key steps in the Wnt signalling pathway. Stabilisation is mediated by Dishevelled family proteins that inhibit the "destruction" complex so that degradation of intracellular β-catenin is reduced, and translocation of β-catenin to the nucleus follows thereafter. Therefore, measuring the level and location of β-catenin in a cell is a good reflection of the level of Wnt/β-catenin signalling. A non-limiting example of such an assay is the "BioImage b-Catenin Redistribution Assay" (Thermo Scientific) which provides recombinant U2OS cells that stably express human β-catenin fused to the C-terminus of enhanced green fluorescent protein (EGFP). Imaging and analysis is performed with a fluorescence microscope or HCS platform allowing the levels and distribution of EGFP-β-catenin to be visualised.

Another way, in which the destruction complex is inhibited, is by removal of Axin by recruitment of Axin to the cytoplasmic tail of the Wnt co-receptor LRP[45, 46]. Axin has been shown to bind preferentially to a phosphorylated form of the LRP tail[47]. Visualisation of Axin translocation, for example with a GFP-Axin fusion protein, is therefore another method for assessing levels of Wnt/β-catenin signalling.

The agonists of the invention may enhance β-catenin signalling by at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%,100%, 110%, 150%, 200%, 250%, 300%, 400% or 500% compared to the β-catenin signalling induced by a neutral substance or negative control as measured in an assay described above, for example as measured in the TOPFlash assay. A compound that does not bind to the Lgr protein, for example an irrelevant IgG1, can be used as a negative control in these assays. The agonists of the invention may enhance β-catenin signalling by a factor of 2x, 5x, 10x,

100x, 1000x, 10000x or more as compared to the activity in the absence of the agonist when measured in an assay described above, for example when measured in the TOPFlash assay, or any of the other assays mentioned herein.

Another method for testing whether a compound has Rspondin-mimicking activity, is to see whether it can rescue organoid growth in Rspondin-depleted culture medium. For example, using the stem cell medium described in Sato et al (*Nature* **459**, 262-5, 2009), organoid growth can be tested with normal medium (comprising Rspondin), Rspondin-depleted medium, and a medium where Rspondin is replaced by the test compound. Rspondin is required for growth and proliferation of the organoids. In an Rspondin-depleted medium the cells do not form organoids. An agonist of the invention mimics the activity of Rspondin in the culture media and thus allows organoid growth.

In one embodiment, an agonist of the invention mimics Rspondin activity when used at about the same concentration as Rspondin (when used in an equivalent way). In another embodiment, an agonist of the invention mimics Rspondin activity when used at about 2x, 5x, 10x, 20x, 50x or 100x greater concentration than Rspondin. In another embodiment, an agonist of the invention mimics Rspondin activity when used at about 2x, 5x, 10x, 20x, 50x or 100x lower concentration than Rspondin.

In general, the agonists of the invention are free of any antagonistic activity. By antagonistic activity, it is meant that a compound prevents or reduces induction of any of the responses induced by Rspondin, including but are not limited to LRP phosphorylation, β-catenin stabilization, Axin translocation, increased expression of Wnt target genes measured in one of the assays described above. Antagonists may reduce induction of any one or more of these responses by 5%, 10%, 15%, 20%, 25%, 30%, 35%, preferably 40%, 45%, 50%, 55%, 60%, more preferably 70%, 80%, 85%, and most preferably 90%, 95%, 99%, or 100% compared to the responses detected in the presence of a neutral substance or negative control.

Binding of the agonist to the Lgr protein

The agonist of the invention may act to mimic the activity of an Rspondin protein binding to an Lgr protein by binding to the Lgr protein, which may be Lgr4, Lgr5 or Lgr6. The agonist may bind to any region of the Lgr protein involved in activating the Lgr protein and enhancing Wnt/β-catenin signalling. A typical Lgr protein comprises the regions: N-terminal domain, leucine-rich repeats (LRRs), CRL-region (or hinge region), transmembrane (TM) regions, exodomains, intracellular domains and the C-terminal domain. These regions are described by the sequences laid out in table 1 below.

Table 1: SEQ ID NOs for the various regions of Lgr4, Lgr5 and Lgr6 polypeptides.

SEQ ID NO	Sequence	Polypeptide	Protein Region
1	MPGPLGLLCFLALGLLGSAG	Lgr4	Leader
2	PSGAAPPLCAAPCSCDGDRRVDCSGKGLTAVPEGLSAFTQA	Lgr4	N-terminal
3	LDISMNNITQLPEDAFKNFPFLEE	Lgr4	LRR1
4	LQLAGNDLSFIHPKALSGLKELKV	Lgr4	LRR2
5	LTLQNNQLKTVPSEAIRGLSALQS	Lgr4	LRR3
6	LRLDANHITSVPEDSFEGLVQLRH	Lgr4	LRR4
7	LWLDDNSLTEVPVHPLSNLPTLQA	Lgr4	LRR5
8	LTLALNKISSIPDFAFTNLSSLVV	Lgr4	LRR6
9	LHLHNNKIRSLSQHCFDGLDNLETL	Lgr4	LRR7
10	DLNYNNLGEFPQAIKALPS	Lgr4	LRR8
11	LKELGFHSNSISVIPDGAFDGNPLLRT	Lgr4	LRR9
12	IHLYDNPLSFVGNSAFHNLSDLHS	Lgr4	LRR10
13	LVIRGASMVQQFPNLTGTVHLES	Lgr4	LRR11
14	LTLTGTKISSIPNNLCQEQKMLRT	Lgr4	LRR12
15	LDLSYNNIRDLPSFNGCHALEE	Lgr4	LRR13
16	ISLQRNQIYQIKEGTFQGLISLR	Lgr4	LRR14
17	ILDLSRNLIHEIHSRAFATLGPITN	Lgr4	LRR15
18	LDVSFNELTSFPTEGLNGLNQLK	Lgr4	LRR16

19	LVGNFKLKEALAAKDFVNLRSLSV	Lgr4	LRR17
20	PYAYQCCAFWGCDSYANLNTEDNSLQDHSVAQEKGTADAANV TSTLENEEHSQIIIHCTPSTGAFKPCEYLLGSWMIR	Lgr4	CRL
21	LTVWFIFLVALFFNLLVILTTF	Lgr4	TM1
22	ASCTSLPSS	Lgr4	intra-1
23	KLFIGLISVSNLFMGIYTGILTFLDA	Lgr4	TM2
24	VSWGRFAEFGIWWETGSGCKV	Lgr4	exo-1
25	AGFLAVFSSESAIFLLMLATVER	Lgr4	TM3
26	SLSAKDIMKNGKSNHLKQ	Lgr4	intra-2
27	FRVAALLAFLGATVAGCFPLFHRGE	Lgr4	TM4
28	YSASPLCLPFPTGETPSL	Lgr4	exo-2
29	GFTVTLVLLNSLAFLLMAVIYTKLY	Lgr4	TM5
30	CNLEKEDLSENSQSSMIK	Lgr4	intra-3
31	HVAWLIFTNCIFFCPVAFFSFAPL	Lgr4	TM6
32	ITAISISPEIM	Lgr4	exo-3
33	KSVTLIFFPLPACLNPVLYVFFNP	Lgr4	TM7
34	KFKEDWKLLKRRVTKKSGSVSVSISSQGGCLEQDFYYDCGMYSHL QGNLTVCDCCESFLLTKPVSCKHLIKSHSCPALAVASCQRPEGYW SDCGTQSAHSDYADEEDSFVSDSSDQVQACGRACFYQSRGFPLV RYAYNLPRVKD	Lgr4	C-terminal
35	PSGAAPPLCAAPCSCDGDRRVDCSGKGLTAVPEGLSAFTQALDIS MNNITQLPEDAFKNFPFLEE	Lgr4	N-terminal + LRR1
36	PSGAAPPLCAAPCSCDGDRRVDCSGKGLTAVPEGLSAFTQALDIS MNNITQLPEDAFKNFPFLEELQLAGNDLSFIHPKALSGLKELKV	Lgr4	N-terminal + LRR1+ LRR2
37	PSGAAPPLCAAPCSCDGDRRVDCSGKGLTAVPEGLSAFTQALDIS MNNITQLPEDAFKNFPFLEELQLAGNDLSFIHPKALSGLKELKVLT LQNNQLKTVPSEAIRGLSALQS	Lgr4	N-terminal + LRR1+ LRR2 + LRR3
38	MDTSRLGVLLSLPVLLQLAT	Lgr5	Leader
39	GGSSPRSGVLLRGCPTHCHCEPDGRMLLRVDCSDLGLSELPSNLS VFTSY	Lgr5	N-terminal
40	LDLSMNNISQLLPNPLPSLRFLEE	Lgr5	LRR1

41	LRLAGNALTYIPKGAFTGLYSLKV	Lgr5	LRR2
42	LMLQNNQLRHVPTEALQNLRSLQS	Lgr5	LRR3
43	LRLDANHISYVPPSCFSGLHSLRH	Lgr5	LRR4
44	LWLDDNALTEIPVQAFRSLSALQA	Lgr5	LRR5
45	MTLALNKIHHIPDYAFGNLSSLVV	Lgr5	LRR6
46	LHLHNNRIHSLGKKCFDGLHSLETL	Lgr5	LRR7
47	DLNYNNLDEFPTAIRTLSN	Lgr5	LRR8
48	LKELGFHSNNIRSIPEKAFVGNPSLIT	Lgr5	LRR9
49	IHFYDNPIQFVGRSAFQHLPELRT	Lgr5	LRR10
50	LTLNGASQITEFPDLTGTANLES	Lgr5	LRR11
51	LTLTGAQISSLPQTVCNQLPNLQV	Lgr5	LRR12
52	LDLSYNLLEDLPSFSVCQKLQK	Lgr5	LRR13
53	IDLRHNEIYEIKVDTFQQLLSLR	Lgr5	LRR14
54	SLNLAWNKIAIIHPNAFSTLPSLIK	Lgr5	LRR15
55	LDLSSNLLSSFPITGLHGLTHLK	Lgr5	LRR16
56	LTGNHALQSLISSENFPELKVIEM	Lgr5	LRR17
57	PYAYQCCAFGVCENAYKISNQWNKGDNSSMDDLHKKDAGMF QAQDERDLEDFLLDFEEDLKALHSVQCSPSPGPFKPCEHLLDGWL IR	Lgr5	CRL
58	IGVWTIAVLALTCNALVTSTVF	Lgr5	TM1
59	RSPLYISPI	Lgr5	intra-1
60	KLLIGVIAAVNMLTGVSSAVLAGVDA	Lgr5	TM2
61	FTFGSFARHGAWWENGVGCHV	Lgr5	exo-1
62	IGFLSIFASESSVFLLTLAALER	Lgr5	TM3
63	GFSVKYSAKFETKAPFSS	Lgr5	intra-2
64	LKVIILLCALLALTMAAVPLLGGSK	Lgr5	TM4
65	YGASPLCLPLPFGEPSTM	Lgr5	exo-2
66	GYMVALILLNSLCFLMMTIAYTKLY	Lgr5	TM5
67	CNLDKGDLENIWDCSMVK	Lgr5	intra-3
68	HIALLLFTNCILNCPVAFLSFSSL	Lgr5	TM6
69	INLTFISPEVI	Lgr5	exo-3

70	KFILLVVVPLPACLNPLLYILFNP	Lgr5	TM7
71	HFKEDLVSLRKQTYVWTRSKHPSLMSINSDDVEKQSCDSTQALV TFTSSSITYDLPPSSVPSPAYPVTESCHLSSVAFVPCL	Lgr5	C-terminal
72	GGSSPRSGVLLRGCPTHCHCEPDGRMLLRVDCSDLGLSELPSNLS VFTSYLDLSMNNISQLLPNPLPSLRFLEE	Lgr5	N-terminal + LRR1
73	GGSSPRSGVLLRGCPTHCHCEPDGRMLLRVDCSDLGLSELPSNLS VFTSYLDLSMNNISQLLPNPLPSLRFLEELRLAGNALTYIPKGAFTG LYSLKV	Lgr5	N-terminal + LRR1+ LRR2
74	GGSSPRSGVLLRGCPTHCHCEPDGRMLLRVDCSDLGLSELPSNLS VFTSYLDLSMNNISQLLPNPLPSLRFLEELRLAGNALTYIPKGAFTG LYSLKVLMLQNNQLRHVPTEALQNLRSLQS	Lgr5	N-terminal + LRR1+ LRR2 + LRR3
75	MPSPPGLRALWLCAALCASRRAGG	Lgr6	Leader
76	APQPGPGPTACPAPCHCQEDGIMLSADCSELGLSAVPGDLDPLT AY	Lgr6	N-terminal
77	LDLSMNNLTELQPGLFHHLRFLEE	Lgr6	LRR1
78	LRLSGNHLSHIPGQAFSGLYSLKI	Lgr6	LRR2
79	LMLQNNQLGGIPAEALWELPSLQS	Lgr6	LRR3
80	LRLDANLISLVPERSFEGLSSLRH	Lgr6	LRR4
81	LWLDDNALTEIPVRALNNLPALQA	Lgr6	LRR5
82	MTLALNRISHIPDYAFQNLTSLVV	Lgr6	LRR6
83	LHLHNNRIQHLGTHSFEGLHNLETL	Lgr6	LRR7
84	DLNYNKLQEFPVAIRTLGR	Lgr6	LRR8
85	LQELGFHNNNIKAIPEKAFMGNPLLQT	Lgr6	LRR9
86	IHFYDNPIQFVGRSAFQYLPKLHT	Lgr6	LRR10
87	LSLNGAMDIQEFPDLKGTTSLEI	Lgr6	LRR11
88	LTLTRAGIRLLPSGMCQQLPRLRVLELSHN	Lgr6	LRR12
89	QIEELPSLHRCQKLEEIGLQHNRI	Lgr6	LRR13
90	WEIGADTFSQLSSLQALDLSWN	Lgr6	LRR14
91	AIRSIHPEAFSTLHSLVKLDLTD	Lgr6	LRR15
92	NQLTTLPLAGLGGLMHLK	Lgr6	LRR16
93	LKGNLALSQAFSKDSFPKLRILEV	Lgr6	LRR17

94	PYAYQCCPYGMCASFFKASGQWEAEDLHLDDEESSKRPLGLLAR QAENHYDQDLDELQLEMEDSKPHPSVQCSPTPGPFKPCEYLFES WIG	Lgr6	CRL
95	RLAVWAIVLLSVLCNGLVLLTVF	Lgr6	TM1
96	AGGPVPLPPV	Lgr6	intra-1
97	KFVVGAIAGANTLTGISCGLLASVDAL	Lgr6	TM2
98	TFGQFSEYGARWETGLGCRAT	Lgr6	exo-1
99	GFLAVLGSEASVLLLTLAAVQC	Lgr6	TM3
100	SVSVSCVRAYGKSPSLGS	Lgr6	intra-2
101	VRAGVLGCLALAGLAAALPLASVGE	Lgr6	TM4
102	YGASPLCLPYAPPEGQPAAL	Lgr6	exo-2
103	GFTVALVMMNSFCFLVVAGAYIKLY	Lgr6	TM5
104	CDLPRGDFEAVWDCAMVRH	Lgr6	intra-3
105	VAWLIFADGLLYCPVAFLSFASM	Lgr6	TM6
106	LGLFPVTPEAV	Lgr6	exo-3
107	KSVLLVVLPLPACLNPLLYLLFNPH	Lgr6	TM7
108	FRDDLRRLRPRAGDSGPLAYAAAGELEKSSCDSTQALVAFSDVDL ILEASEAGRPPGLETYGFPSVTLISCQQPGAPRLEGSHCVEPEGNH FGNPQPSMDGELLLRAEGSTPAGGGLSGGGGFQPSGLALLHTYE FCRYPAQWRPLESRGPV	Lgr6	C-terminal
109	APQPGPGPTACPAPCHCQEDGIMLSADCSELGLSAVPGDLDPLT AYLDLSMNNLTELQPGLFHHLRFLEE	Lgr6	N-terminal + LRR1
110	APQPGPGPTACPAPCHCQEDGIMLSADCSELGLSAVPGDLDPLT AYLDLSMNNLTELQPGLFHHLRFLEELRLSGNHLSHIPGQAFSGLY SLKI	Lgr6	N-terminal + LRR1+ LRR2
111	APQPGPGPTACPAPCHCQEDGIMLSADCSELGLSAVPGDLDPLT AYLDLSMNNLTELQPGLFHHLRFLEELRLSGNHLSHIPGQAFSGLY SLKILMLQNNQLGGIPAEALWELPSLQS	Lgr6	N-terminal + LRR1+ LRR2 + LRR3

In one embodiment, the agonist binds to the extracellular parts or transmembrane regions of the Lgr protein. In a further embodiment, the agonist binds to the extracellular parts of

the Lgr protein comprising the N-terminal region, any of the 17 leucine rich repeats, the CRL region and/or any of the 3 exodomains (as designated exo-1, exo-2 and exo-3 in table 1).

In one embodiment, the agonist may bind to one or more of the leucine-rich repeats (LRRs) of the Lgr protein. For example, the agonist may bind a region consisting of or comprising LRR1, LRR2, LRR3, LRR4, LRR5, LRR6, LRR7, LRR8, LRR9, LRR10, LRR11, LRR12, LRR13, LRR14, LRR15, LRR16, and/or LRR17 of any of the Lgr4, Lgr5 or Lgr6 polypeptides, as shown in Table 1 above, or to an epitope within these regions. Alternatively, the agonist may bind to epitopes within a region consisting of or comprising the N-terminus plus LRR1-3, or N-terminus plus LRR1 and LRR2, or N-terminus plus LRR1 of any of the Lgr4, Lgr5 or Lgr6 polypeptides, as shown in Table 1 above. As explained below, the inventors have shown that Rspondin binds to the LRRs of Lgr proteins and therefore, agonists that mimic Rspondin activity may also bind to this region. Alternatively, the agonist may bind to a conformational epitope that may be induced upon recruitment of the Lgr protein to the Wnt/Frz/LRP complex.

In an alternative embodiment, the agonist of the invention may bind to the extracellular domain or external transmembrane loops of the Lgr protein or to an epitope within these regions. The Lgr protein is a 7 transmembrane domain protein and the agonist may bind to a region of the Lgr protein that is extracellular in the native state of the protein, or to an epitope within these regions. For example, in one embodiment, the agonist may bind to an epitope within the CRL-region of any of the Lgr4, Lgr5 or Lgr6 polypeptides, as shown in Table 1 above. Another possibility is that the agonist of the invention may bind to any one of the three exodomains or combinations thereof that are located between the transmembrane regions of the Lgr peptide. For example, the agonist of the invention may bind to an epitope within a region of the protein consisting of or comprising exo-1, exo-2, and/or exo-3 of any of the Lgr4, Lgr5 or Lgr6 polypeptides, as shown in Table 1 above.

The inventors have shown that antibody 1D9 mimics Rspondin activity and is therefore an agonist (see Example 3). Figure 26 shows that antibody 1D9 does not bind to Lgr5-

478, which represents an Lgr5 fragment comprising the exo domain of Lgr5 without the CRL region. However, antibody 1D9 does bind to Lgr5-543, which represents the full exo domain of Lgr5 (the CRL and the LRRs). Therefore, the inventors conclude that antibody 1D9 binds to the CRL region of Lgr5. SEQ ID NO: 57 represents the CRL region (or hinge region) of Lgr5. The inventors have thus identified a region on Lgr5 which is involved in the activation of Wnt signalling by Lgr5. This permits the design and screening of agonists that target a region of Lgr5 comprising or consisting of the whole or part of the CRL region i.e. comprising or consisting of the whole or part of SEQ ID NO:57 (see section on "Methods for identifying further agonists of the invention").

Therefore, in one embodiment, an agonist of the invention binds to a region consisting of or comprising the CRL (or hinge region) of Lgr4, Lgr5 and/or Lgr6, or i.e. the region represented by SEQ ID NOs: 20, 57 and/or 94 respectively. In some embodiments an agonist of the invention binds to the region of Lgr5 represented by SEQ ID NO: 57 but does not bind to the region of Lgr4 represented by SEQ ID NO: 20 or the region of Lgr6 represented by SEQ ID NO: 94. In some embodiments an agonist of the invention binds to the region of Lgr4 represented by SEQ ID NO: 20 but does not bind to the region of Lgr5 represented by SEQ ID NO: 57 or the region of Lgr6 represented by SEQ ID NO: 94. In some embodiments an agonist of the invention binds to the region of Lgr6 represented by SEQ ID NO: 94 but does not bind to the region of Lgr4 represented by SEQ ID NO: 20 or the region of Lgr5 represented by SEQ ID NO: 57.

In some embodiments the agonist is an antibody that does not bind the CRL region of Lgr4, Lgr5 and/or Lgr6 i.e. the region represented by SEQ ID NOs: 20, 57 and/or 94 respectively.

The inventors have also demonstrated that antibody 1D9 and Rspondin bind to separate regions on Lgr5. Figure 26 demonstrates that both antibody 1D9 and Rspondin can bind to Lgr5-543 (full exo domain of Lgr5 i.e. CRL and LRRs) at the same time to form a complex. Figure 26 also shows that Rspondin binds to Lgr5-478 which represents the exo domain without the CRL region. Therefore, the inventors conclude that Rspondin

binds to the LRRs on Lgr proteins. It is surprising that agonistic antibody 1D9 binds to a region on Lgr5 that differs from the region that Rspondin binds to.

Another possibility is that the agonist of the invention may bind to one or more transmembrane region of the Lgr peptide. For example, the agonist of the invention may bind to a region of the protein consisting of or comprising TM1, TM2, TM3, TM4, TM5, TM6, and/or TM7 of any of the Lgr4, Lgr5 or Lgr6 polypeptides, as shown in Table 1 above, or to an epitope within these regions. For example, in one embodiment, the agonist may be a small hydrophobic molecule that binds in the transmembrane region and disrupts protein-lipid interactions, causing favourable conformational or biochemical changes which result in agonistic activity.

In a further embodiment, the agonist may be a competitive agonist that binds to the Rspondin binding site in the Lgr protein. The Rspondin binding site is typically located at the N-terminus of the Lgr protein. The N-terminus, leucine-rich repeat 1 and leucine-rich repeat 2 (this region is described by SEQ ID NOs: 36, 73, 110 for Lgr4, Lgr5 and Lgr6 respectively) are essential for binding of Rspondin1-4 to of any of the Lgr4, Lgr5 or Lgr6 polypeptides. It is predicted that this holds true for all three Lgrs and all four Rspondins based on sequence homology. The agonist may bind to an epitope within an Rspondin binding site on an Lgr protein, in particular to at least 1, 2, 3, 4, 5, 6 or more amino acids within the Rspondin binding site.

Such competitive agonists can be identified by standard methods known in the art. For example, the skilled person might identify agonists that compete for Rspondin binding in a competitive ELISA assay. Preferred competitive agonists bind to the Lgr protein with a greater affinity and/or avidity than that of Rspondin. Assays for identifying compounds that bind to the Lgr proteins are described in more detail below in connection with methods of screening for agonists of the invention.

An epitope is the site on the antigen to which a binding agent, such as for example a ligand or an antibody, binds. If the antigen is a polymer, such as a protein or polysaccharide, the epitope can be formed by contiguous residues or by non-contiguous residues brought into close proximity by the folding of an antigenic polymer. In proteins,

epitopes formed by contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by noncontiguous amino acids are typically lost under said exposure.

The agonist of the invention may bind to any epitope within these regions of the Lgr proteins, in particular to any amino acid sequence of at least 1, 2, 3, 4, 5, 6 or more amino acids within these regions. The agonists may bind to epitopes within one or more of these regions, either simultaneously or separately. By simultaneously, it is meant that the compound of the invention can bind to epitopes within two or more regions of the Lgr protein at the same time. By separately, it is meant that the compound can only be bound to one epitope within an Lgr region at a single moment in time, but also has capacity to bind an epitope in another region at a different time.

The ability of an agonist of the invention to bind to an Lgr protein can be determined by standard experimental techniques known in the art including immunoprecipitation, western blot, biacore (IBIS), FACS, and mass spectrometry.

The agonist of the invention typically binds to the Lgr protein with a Kd equal to or less than about 10^{-7}M, 10^{-8}M, 10^{-9}M, 10^{-10}M, 10^{-11}M, 10^{-12}M, 10^{-13}M or more. Such values can be determined by binding assays, for example, by surface plasmon resonance or the Kinexa method, as practised by those of skill in the art.

In one aspect of the invention, the agonist binds specifically to the Lgr protein. The term "specific" means that the compound has substantially greater affinity for Lgr polypeptides than their affinity for other related polypeptides. For example, a compound that is specific for Lgr4 will have substantially greater affinity for Lgr4 than for Lgr5 or Lgr6. By "substantially greater affinity" it is meant that there is a measurable increase in the affinity for an Lgr protein as compared with the affinity for other known receptor proteins. In one embodiment, an agonist of the invention is specific for Lgr4, Lgr5 and Lgr6. This means that it has substantially greater affinity for all of these Lgr polypeptides than for other related polypeptides. In another embodiment, an agonist of the invention is specific for only one or two of Lgr4, Lgr5 and/or Lgr6. This means that it has substantially greater affinity for one or two Lgr polypeptides than for another Lgr

polypeptide. Therefore, an agonist of the invention that is specific for Lgr5, need not bind and activate only Lgr5. However, in some embodiments, the agonist of the invention that is specific for Lgr5 preferentially binds and/or activates Lgr5 more than Lgr4 or Lgr6. For example, an agonist may preferentially bind to Lgr5 compared to Lgr4 and Lgr6. Alternatively, it may bind to Lgr4, Lgr5 and Lgr6 with equal affinity but preferentially activate Lgr5 more than the others. The same definition applies for an agonist of the invention that is specific for Lgr4 or Lgr6. In other embodiments, an agonists may bind to and activate only a single class of Lgr molecule i.e. only to Lgr4, Lgr5 or Lgr6.

Exemplary agonists

Small molecules

Agonists of the invention, in particular agonists that bind to Lgr proteins according to the invention may exist in various forms, including natural or modified substrates, enzymes, receptors, small organic molecules, such as small natural or synthetic organic molecules of up to 2000Da, preferably 800Da or less, peptidomimetics, inorganic molecules, peptides, polypeptides, aptamers, and structural or functional mimetics of these including small molecules.

In some embodiments of the invention, the agonist is a modified substrate. For example the modified substrate may comprise or consist of an Rspondin fragment, mutant or derivative that binds Lgr4, Lgr5 and/or Lgr6.

Therefore, in some embodiments, the agonist of the invention is a fragment of Rspondin. Fragments of Rspondin might have advantages over full-length Rspondin. For example, smaller protein fragments are easier to generate by recombinant methods and are more likely to be more stable because they have fewer enzyme cutting sites. Fragments are also more likely to be lacking regulatory domains that may make dosing unpredictable. The use of an Rspondin fragment may be preferable over a full length Rspondin protein, because smaller fragments have enhanced stability in serum, and because fragments

generated by recombinant techniques are easier than full length Rspondin proteins to produce in *E. coli*.

In some embodiments of the invention, an Rspondin fragment comprises at least 50, at least 100, at least 150 or at least 200 consecutive amino acids of SEQ ID NOs: 112, 113, 114 or 115 or of sequences with more than 70, 80, 90 or 99% identity to any one of SEQ ID NOs: 112, 113, 114 or 115. An Rspondin fragment is by definition at least one amino acid shorter than full length wild-type Rspondin. Therefore, an Rspondin fragment is less than 263 amino acids of SEQ ID NO: 112, less than 243 amino acids of SEQ ID NO: 113, less than 272 amino acids of SEQ ID NO: 114, or less than 234 amino acids of SEQ ID NO: 115. In some embodiments the Rspondin fragment comprises or consists of less than 220, less than 200, less than 180, less than 160, less than 140, less than 120, less than 100, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20 consecutive amino acids of SEQ ID NOs: 112, 113, 114 or 115 or of sequences with more than 70, 80, 90 or 99% identity to any one of SEQ ID NOs: 112, 113, 114 or 115.

Full length, wild-type Rspondin proteins comprise a Furin domain, a Thrombospondin domain and a region of basic amino acid-rich repeats (Dev Cell 2004, 7, 525-534, Kazanskaya et al). The Furin domain consists of SEQ ID NOs: 116, 117, 118 and 119 in Rspondin 1, 2, 3 and 4 respectively. The Thrombospondin domain consists of SEQ ID NOs: 120, 121, 122 and 123 in Rspondin 1, 2, 3 and 4 respectively. The region of basic amino acid-rich repeats consists of SEQ ID NOs: 124, 125, 126 and 127 in Rspondin 1, 2, 3 and 4 respectively. The inventors have discovered for the first time that truncated Rspondin proteins without the basic amino acid-rich repeats (SEQ ID NO: 139) and truncated Rspondin proteins without the basic amino acid-rich repeats and without the thrombospondin domain (SEQ ID NO: 140), can still bind to Lgr proteins (for example, see Example 2). Furthermore, the inventors have shown that the "Furin domain fragments" (lacking Thromospondin and the amino acid-rich repeats and represented by SEQ ID NO: 140) are more potent at enhancing the Wnt pathway than full-length Rspondin at the same concentration (see Figure 22).

Thus, in some embodiments, the Rspondin fragment does not comprise at least part of the Thrombospondin domain and/or at least part of the regions of basic amino acid-rich repeats. In some embodiments, the Rspondin fragment does not comprise the Thrombospondin domain and/or the region of basic amino acid-rich repeats.

In some embodiments, the Rspondin fragment is the fragment represented by SEQ ID NO: 139 or SEQ ID NO: 140.

Furthermore, the inventors have shown that an anti-Rspondin 3 monoclonal antibody, which binds to epitopes in the Furin domain of Rspondin 3, blocks the Wnt-enhancing activity of Rspondin (for example, see Figure 21). Therefore, the inventors conclude that the Furin domain is sufficient and necessary for binding and for the activity of Rspondin. The skilled person could not have expected that the Furin domain alone would be necessary for Rspondin binding to Lgr proteins and sufficient for mimicking the Wnt-enhancing activity of Rspondin.In one embodiment of the invention, the Rspondin fragment comprises or consists of an Rspondin Furin domain, for example selected from SEQ ID NOs: 116, 117, 118, 119, 140, or 143, and an N-terminal domain. In a further embodiment of the invention, the Rspondin fragment comprises or consists of an Rspondin Furin domain. The Rspondin Furin domain may have more than 70, 80, 90 or 99% identity to SEQ ID NOs: 116, 117, 118, 119, 140, or 143. The Rspondin Furin domain may consist of SEQ ID NOs: 116, 117, 118, 119, 140, or 143. Alternatively, the Rspondin fragment comprises at least 50, at least 60, at least 70, at least 80 or at least 90 consecutive amino acids of SEQ ID NOs: 116, 117, 118, 119, 140 or 143. In some embodiments the Rspondin Furin domain fragment comprises or consists of less than 100, less than 90, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20, less than 10 consecutive amino acids of SEQ ID NOs: 116, 117, 118, 119, 140, or 143 or of sequences with more than 70, 80, 90 or 99% identity to any one of SEQ ID NOs: 116, 117, 118, 119, 140, or 143.

Sequence identity between polypeptide sequences is preferably determined by pairwise alignment algorithm using the Needleman-Wunsch global alignment algorithm (Needleman& Wunsch (1970) *J. Mol. Biol.* 48, 443-453), using default parameters (*e.g.*

with Gap opening penalty = 10.0, and with Gap extension penalty = 0.5, using the EBLOSUM62 scoring matrix). This algorithm is conveniently implemented in the *needle* tool in the EMBOSS package (Rice *et al.* (2000) *Trends Genet* 16:276-277). Sequence identity should be calculated over the entire length of the polypeptide sequence of the invention.

In a further embodiment of the invention, the Rspondin fragment that comprises or consists of an Rspondin Furin domain, additionally comprises of an Rspondin Thrombospondin domain, for example as shown in SEQ ID NO: 139.

In some embodiments of the invention, the agonist may comprise more than one Rspondin fragment from either the same or one or more different Rspondin proteins. These fragments may be joined together in any order e.g. by genetic recombination or by a linking molecule. In a preferred embodiment, the fragments may be joined in the same order that they might appear in a full length wild-type Rspondin protein. For example, the agonist may comprise the Furin domain of Rspondin 1 and the Thrombospondin domain of Rspondin 2, 3 or 4, or vice versa. The skilled person will appreciate that many combinations of Rspondin 1, 2, 3 and 4 fragments are possible and that many combinations would be expected to result in fully functional Rspondin proteins.

In some embodiments, the agonist is a mutant Rspondin or Rspondin derivative that is specific to only Lgr4, Lgr5 or Lgr6. For example in some embodiments the agonist is an Rspondin derivative specific to only Lgr5, for example by mutation of one or more amino acid residues involved in binding to the Lgr4, Lgr5 and Lgr6 proteins.

In some embodiments of the invention, an Rspondin derivative may be, for example, Rspondin1, Rspondin2, Rspondin3 or Rspondin4 with one or more amino acid mutations, including substitutions, insertions or deletions, that cause it to have increased specificity for one class of Lgr molecule compared to another. Identification of such amino acid substitutions, insertions or deletions could be determined by protein engineering techniques known in the art. For example, an Rspondin derivative may be engineered to have increased specificity for Lgr5 compared to Lgr4 or Lgr6. Alternatively, an Rspondin derivative may bind and activate only Lgr5 and not Lgr4 or

Lgr6. In an alternative embodiment, the Rspondin derivative is specific for, or has greater specificity for Lgr4 (relative to Lgr5 and/or Lgr6) or Lgr6 (relative to Lgr4 and/or Lgr5). This would be advantageous for use in therapy, for example, because wild-type Rspondin binds to Lgr4, Lgr5 and Lgr6 and thus stimulates growth, via the Wnt pathway, in all proliferating cells. An Rspondin derivative that has increased specificity for Lgr5, would preferentially stimulate growth of Lgr5-positive stem cells, not in all dividing cells. Therefore, in situations where wild-type Rspondin causes too much regrowth, an Rpsondin derivative with specificity for only a single Lgr protein, for example Lgr5 or Lgr6, would be more appropriate for regenerative treatments.

An example of a variant of Rspo1 is shown in SEQ ID NO: 128, and has a single mutation V50I. In one embodiment of the invention the Rspondin derivative is not the variant of Rspo1 that is shown in SEQ ID NO: 128.

In some embodiments, the agonist of the invention is not an Rspondin protein. In some embodiments, the agonist of the invention is not a fragment of an Rspondin protein. In some embodiments, the agonist of the invention is not a derivative of Rspondin. In some embodiments, the agonist of the invention is not a furin domain fragment. This is particularly envisaged in some embodiments relating to the agonist *per se*. However, in other aspects of the invention, it is envisaged that an Rspondin protein, fragment or derivative, for example a furin domain fragment is encompassed by the invention, for example wherein the agonist is a multi-targeting compound and/or when the agonist or multi-targeting compound is present/used in for example pharmaceutical compositions, multi-targeting compounds, methods for treatment, methods for enhancing cell growth/proliferation and methods for identifying new agonists.

In one embodiment of the invention, the agonist is an aptamer. As used herein, the term "aptamer" refers to strands of oligonucleotides (DNA or RNA) that can adopt highly specific three-dimensional conformations. Aptamers are designed to have high binding affinities and specificities towards certain target molecules, including extracellular and intracellular proteins.

Antibodies

In another embodiment, the agonist is an antibody.

A conventional antibody is comprised of two identical heavy chains and two identical light chains that are joined by disulfide bonds. Each heavy and light chain contains a constant region and a variable region. Each variable region contains three CDRs which are primarily responsible for binding an epitope of an antigen, in this case the Lgr protein, as discussed above. They are referred to as CDR1, CDR2, and CDR3, numbered sequentially from the N-terminus, of which the CDR3 region comprises the most variable region and normally provides a substantial part of the contact residues to a target. The more highly conserved portions of the variable regions are called the "framework regions".

The term antibody is used herein in the broadest sense and specifically covers, but is not limited to, monoclonal antibodies (including full length monoclonal antibodies) of any isotype such as IgG, IgM, IgA, IgD and IgE, polyclonal antibodies including recombinant polyclonal antibodies, Oligoclonics, multispecific antibodies, chimeric antibodies, nanobodies, diabodies, BiTE's, Tandabs, mimetobodies, bispecific antibodies, humanized antibodies, human antibodies, deimmunised antibodies and antibody fragments. In addition, scaffolds will be covered under this term, such as Anticalins, Ankarins, etc. An antibody reactive with a the specific epitopes of the Lgr proteins discussed above can be generated by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors, or by immunizing an animal with the Lgr epitopes of nucleic acid encoding them.

Antibodies of the invention will have binding properties described above for agonists in general. Antibody characteristics, such as on-rates (ka), off-rates (kd) and affinities (KD) can be determined in competitive binding assays using known platforms such as Octet™ (ForteBio), ProteOn™ (Bio-Rad), and Biacore™ (GE Healthcare). A population of monoclonal antibodies consists of antibodies directed against a single epitope. The monoclonal antibodies are essentially identical, in general because they are made by identical immune cells that are all clones of a particular parent cell. There is a possibility

that naturally occurring mutations may cause differences between monoclonal antibodies in the same population in minor amounts. Monoclonal antibodies to Lgr proteins that mimic the activity of Rspondin by binding to the Lgr protein, e.g. by binding the agonistic epitopes discussed above, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies using hybridoma technology is well known. In addition antibodies of this type can be generated by phage display technology or ribosome display technology. Panels of monoclonal antibodies produced against the Lgr proteins can be screened to confirm that they are agonists, as well as for various properties, such as isotype, epitope, affinity, etc.

In one embodiment, an antibody according to the invention comprises a single domain antibody, a F(ab')2, Fab, Fab', Facb, or single chain Fv (scFv) fragment. An Fc fragment, which for example activates complement and may bind to Fc receptors, can be present but is not required for an antibody and variants or derivatives thereof. A scFv fragment is an epitope-binding fragment that contains at least one fragment of an antibody heavy chain variable region (VH) linked to at least one fragment of an antibody light chain variable region (VL). The linker may be a short, flexible peptide selected to assure that the proper three-dimensional folding of the VL and VH regions occurs once they are linked so as to maintain the target molecule binding-specificity of the whole antibody from which the single-chain antibody fragment is derived. The carboxyl terminus of the VL or VH sequence may be covalently linked by a linker to the amino acid terminus of a complementary VL or VH sequence.

The antibody may be a nanobody, and/or a bispecific antibody. A nanobody is a single domain antibody that occurs naturally in camelids. In contrast to standard antibodies, nanobodies are relatively simple proteins comprising only a heavy chain- like variable region. Bispecific antibodies are artificially engineered monoclonal antibodies that consist of two distinct binding sites and are capable of binding two different epitopes. Examples of bispecific antibodies are discussed in more detail below in the section on dual-targeting and multi-targeting agonists.

The antibody may be a chimeric antibody comprising a binding portion, for example the variable region or part thereof of the heavy and light chains, of a non-human antibody,

while the remainder portion, for example the constant region of the heavy and light chains, is of a human antibody. A chimeric antibody may be produced by recombinant processes well known in the art, and has an animal variable region and a human constant region.

The antibody may be a human antibody. The term "human antibody" means an antibody in which the variable and constant domain sequences are derived from human sequences. A human antibody can be made by several different ways, including by use of human immunoglobulin expression libraries (Stratagene Corp., La Jolla, California) to produce fragments of human antibodies VH, VL, Fv, Fd, Fab, or (Fab')2, and using these fragments to construct whole human antibodies using techniques similar to those for producing chimeric antibodies. Alternatively, these fragments may be used on their own. Human antibodies can also be produced in transgenic mice with a human immunoglobulin genome. Such mice are available from Abgenix. Inc., Fremont, California, Kyowa Hakko Kirin (KM mouse), Ablexis (AlivaMab mouse) and Medarex, Inc., Annandale, New Jersey. Hybridomas can be generated by conventional procedures by fusing B lymphocytes from the immunized animals with myeloma cells (e.g., Sp2/0 and NSO), as described by Kohler, G. and Milstein, C. 1975[48].

In a humanized antibody, only the complementarity determining regions (CDRs), which are responsible for antigen binding and specificity are animal derived and have an amino acid sequence corresponding to the animal antibody, and substantially all of the remaining portions of the molecule (except, in some cases, small portions of the framework regions within the variable region) are human derived and correspond in amino acid sequence to a human antibody[49, 50]. Methods for humanizing non-human antibodies are known in the art. As is known to the skilled person, antibodies such as rat antibodies can be humanized by grafting their CDRs onto the variable light (VL) and variable heavy (VH) frameworks of human Ig molecules, while retaining those rat framework residues deemed essential for specificity and affinity[51]. Overall, CDR grafted antibodies consist of more than 80% human amino acid sequences[52, 53]. Despite these efforts, CDR-grafted, humanized antibodies were shown to still evoke an antibody response against the grafted V region[54].

A deimmunised antibody is an antibody in which the T and B cell epitopes have been eliminated. They have reduced immunogenicity when applied *in vivo*. To further decrease the content of non-human sequences in therapeutic mAbs, humanization methods based on different paradigms such as resurfacing[55], superhumanization[56], human string content optimization[57] and humaneering have been developed[58]. As in CDR grafting approaches, these methods rely on analyses of the antibody structure and sequence comparison of the non- human and human mAbs in order to evaluate the impact of the humanization process into immunogenicity of the final product.

De-immunization is another approach developed to reduce the immunogenicity of chimeric or rat antibodies. It involves the identification of linear T-cell epitopes in the antibody of interest, using bioinformatics, and their subsequent replacement by site-directed mutagenesis to human or non- immunogenic sequence[59]. Although de-immunized antibodies exhibited reduced immunogenicity in primates, compared with their chimeric counterparts, some loss of binding affinity was observed[60].

Antibodies that are chimeric, deimmunised, humanized, human-like, resurfaced or humanized monoclonal or human antibodies are particular useful for treatment protocols because such antibodies can reduce immunogenicity and thus avoid human anti-mouse antibody (HAMA) response. In certain conditions it is preferable that the antibody is virtually free of Fc effector function, such is for example the case for natural IgG4 or IgG2, or for genetically mutated IgG or IgM which does not augment antibody-dependent cellular cytotoxicity[61] and complement mediated cytolysis[62, 63]. In addition, in case of IgG4, it is preferred to use stabilized hinge forms of IgG4 to prevent the occurrence of half molecule formation[64] or other forms of IgG4 aimed at preventing half molecule formation. In other conditions, it is desired that antibodies have enhanced Fc effector function. Such enhanced Fc effector function can be achieved, for example, by Fc engineering. One example of technology used for Fc-engineering is the Xmab® Technology of Xencor (www.xencor.com).

Antibodies are glycoproteins containing between 3 and 12% carbohydrate. The carbohydrate units are transferred to acceptor sites on the antibody chains after the heavy

and light chains have combined. The major carbohydrate units are attached to amino acid residues of the constant region of the antibody. The carbohydrate units may affect overall solubility and the rate of catabolism of the antibody. It is also known that carbohydrate is necessary for cellular secretion of some antibody chains. It has been demonstrated that glycosylation of the constant region plays a vital role in the effector functioning of an antibody; without this glycosylation in its correct configuration, the antibody may be able to bind to the antigen but may not be able to bind for example to macrophages, helper and suppressor cells or complement, to carry out its role of blocking or lysing the cell to which it is bound. The antibody of the invention may therefore be glycosylated in the cells in which it is produced to maintain antigen binding capability and effector functionality.

Antibodies produced according to a method of the invention may further differ in acetylation, pegylation, phosphorylation, and/or amidation, compared to the antibody produced by the hybridoma cell.

Antibodies of the invention, whether polyclonal or monoclonal, may have additional utility in that they may be employed as reagents in immunoassays, radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA) or protein arrays. In these applications, the antibodies can be labelled with an analytically-detectable reagent such as a radioisotope, a fluorescent molecule or an enzyme.

An example of an agonistic antibody is the antibody 1D9 (see Example 3). Therefore, in one embodiment, the agonist is the antibody 1D9 (also referred to herein as Ab1). The VL of antibody 1D9 is represented by SEQ ID NO: 131 and the VH is represented by SEQ ID NO: 129. Therefore, in one embodiment, the agonist is an antibody comprising or consisting of SEQ ID NO: 129 and/or SEQ ID NO: 131. The CDRs of antibody 1D9 are represented by SEQ ID NOs: 133-138. A hybridoma producing 1D9 has been deposited at the institute BCCM / LMBP on 28/07/2009 with the following accession number: LMBP 6961CB.

In one embodiment the agonist is an antibody (or fragment or derivative thereof), wherein the sequence of the VH is at least 90%, at least 95%, at least 98%, or at least

99% identical to SEQ ID NO: 129 and/or the sequence of the VL is at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 131.

In some embodiments the agonist is a chimeric, deimmunised, humanized, human-like, resurfaced or humanized monoclonal or human antibody (or fragment or derivative thereof) wherein the sequence of the VH is at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 129 and/or the sequence of the VL is at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 131.

In one embodiment the agonist is an antibody (or fragment or derivative thereof), wherein the CDRs of said antibody (or fragment or derivative thereof) comprise one or more, preferably all of the CDR sequences of the 1D9 antibody or sequences that are at least 90%, at least 95%, at least 98%, or at least 99% identical to those sequences.

In some embodiments the agonist is a chimeric, deimmunised, humanized, human-like, resurfaced or humanized monoclonal or human antibody (or fragment or derivative thereof) wherein the CDRs of said antibody (or fragment or derivative thereof) comprise one or more, preferably all of the CDR sequences of the 1D9 antibody or sequences that are at least 90%, at least 95%, at least 98%, or at least 99% identical to those sequences.

Antibody 1D9 binds to the CRL region on Lgr5 (see section on "Binding of the agonist to the Lgr protein"). Other antibodies targeting the CRL region on Lgr4, Lgr5 or Lgr6 cells could also be expected to have agonistic activity. Therefore, in some embodiments of the invention, the agonist is an antibody that binds to the CRL region of an Lgr protein.

In some embodiments of the invention, the agonist of the invention is not antibody 1D9. This is particularly envisaged in some embodiments relating to the agonist *per se*. However, in other aspects of the invention, it is envisaged that 1D9 is encompassed by the invention, for example wherein the agonist is a multi-targeting compound and/or when the agonist or multi-targeting compound is present/used in for example pharmaceutical compositions, multi-targeting compounds, methods for treatment,

methods for enhancing cell growth/proliferation and methods for identifying new agonists.

Multi-targeting compounds

In some embodiments, the agonist of the invention is a multi-targeting compound as described herein. By "multi-targeting compound", it is meant that the agonist of the invention additionally binds to at least one further target polypeptide or other target molecule. In some embodiments the other target molecule is a carbohydrate moiety. Therefore, in some embodiments the invention provides a multi-targeting compound, comprising at least one portion with Rspondin-mimicking activity, and at least one other portion that binds to a further target polypeptide or other target molecule. The phrases "agonist which is a multi-targeting compound" and "agonist of the invention which additionally binds to at least one further target polypeptide or other target molecule " are used interchangeably herein. For example, the agonist may bind to an Lgr protein and thus mimic the activity of Rspondin and additionally binding at least a second polypeptide or other target molecule, for example to confer tissue-specific targeting of the Rspondin-mimicking activity. In some aspects, the agonist may bind 2, 3, 4 or more polypeptides.

The further target polypeptide or other target molecule may for example be a Frizzled Receptor, for example Frz1, Frz2, Frz3, Frz4, Frz5, Frz6, Frz7, Frz8, Frz9 or Frz10. In one embodiment, the agonist may bind i) at least one of Lgr4, Lgr5 or Lgr6, and ii) at least one of Frz1-10. In particular, the agonist may bind i) at least one of Lgr4, Lgr5 or Lgr6, and ii) at least one of Frz5, Frz6 and Frz7. Such a compound might modulate activity of the Wnt pathway via both Lgr and Frz in the Wnt receptor complex, for example it may enhance Wnt signalling to a greater extent by mimicking the natural synergistic action of Rspondin and soluble Wnt molecules in vertebrate cells.

The further target polypeptide or other target molecule may be an LRP, for example LRP5 or LRP6. In one embodiment, the agonist may bind i) at least one of Lgr4, Lgr5 or Lgr6, and ii) at least one of LRP5-6. Such a compound might modulate activity of the Wnt pathway via both Lgr and LRP in the Wnt receptor complex. For example it may

enhance Wnt signalling to a greater extent by mimicking the natural synergistic action of Rspondin and soluble Wnt molecules in vertebrate cells.

In an alternative embodiment, the further target polypeptide or other target molecule may be a second Lgr protein for example Lgr 4, 5 or 6, such that the agonist targets two or three Lgr proteins. For example, an agonist of the invention might bind Lgr4 and Lgr5, Lgr5 and Lgr 6, or Lgr4 and Lgr6, or Lgr4, Lgr5 and Lgr6. The combination of Lgr4 and Lgr5 antibodies in a mixture or as a bispecific may be particularly useful because the inventors have found that in many tissues Lgr5 expression is usually accompanied by Lgr4 expression (see Example 1). Therefore, an agonist that binds to both Lgr4 and Lgr5 may have a greater effect on enhancing Wnt signaling in these tissues.

Alternatively, the further target polypeptide or other target molecule might be a tissue-specific, or cell-specific marker for tissue-specific or cell-specific targeting. The agonist of the invention may thus bind an Lgr protein and a tissue-specific, or cell-specific marker. For example, for liver-specific targeting, an agonist of the invention may bind Lgr and L-cadherin. The agonist might also bind a further target polypeptide or other target molecule present in epithelial tissues, such as skin tissue, stomach lining, pancreatic lining, liver, kidney; connective tissues, such as inner layers of skin, tendons, ligaments, cartilage, bone, fat, hair, blood; muscle tissues; and nerve tissues, such as glial cells and neurons. Such markers include, but are not limited to membrane proteins that are specifically expressed on certain organs such as liver, pancreas or colon. These markers are well known in the art.

In other embodiments, the further target polypeptide or other target molecule may be a tissue-specific marker that is expressed on cells in the liver, pancreas, kidney, small intestine, colon etc. Such markers include, but are not limited to Epcam for epithelial cells, CA19 for pancreas, A33 for intestine, L-cadherin for the liver, dipeptidyl peptidase V for the pancreas and/or liver, L-SIGN for endothelial cells of the liver and lymph nodes (Gardner et al., 100(8) 4498-4503 PNAS 2002), CD-26 (also known as DPPIV) for the liver (in particular for ducts and hepatocytes), Integrin a6b1 for the liver (in

particular for biliary ducts) (Couvelard et al., Hepatology 27(3), 839-847 (1998)). Therefore, a multi-targeting compound of the invention may target Lgr4 (which is expressed by all proliferating cells) and/or Lgr5 and/or Lgr6, and one or more of these tissue-specific markers.

In an alternative embodiment, the further target polypeptide or other target molecule may be a secreted molecule that is itself naturally (or otherwise) targeted to a tissue or cell of interest. For example, coagulation factor IX and complement component C4 are secreted molecules that are targeted to the liver.

In one embodiment, the multi-targeting compound has the effect of increasing the affinity of the Rspondin-mimicking activity for the target cell and/or tissue. An agonist used alone will diffuse throughout its environment (e.g. the body or cell culture) where it is used. This means that it will activate all cells with an Rspondin-responsive receptor (now known to include Lgr4, Lgr5 and Lgr6). Where Rspondin might be used for treatment purposes (such as those described later in the application) and is administered to an animal or patient, the dose would have to be kept low in order to minimise adverse effects on non-target organs/tissues/cells caused by non-specific binding of Rspondin to its receptors. Agonists that bind to a second or third (or more) target polypeptides that are specific for the target organ/tissue/cell, would require lower dosages to achieve similar, equal or better activation of the Wnt pathway in a target organ/tissue/cell whilst minimising the adverse effects on the non-target organs/tissues/cells. Therefore, in one embodiment, the further target polypeptide or other target molecule is a membrane molecule that is expressed on the target organ/tissue/cell of interest and optionally is not expressed on other non-target organs/tissues/cells and optionally not expressed on cells which do not have Rspondin-responsive receptors.

The present inventors have also surprisingly discovered that Lgr proteins are expressed basolaterally (see Figure 27). Therefore, it is clearly advantageous to target a multi-targeting agonist to other basolaterally expressed targets. If the multi-targeting compound was targeted to the apical side of a tissue membrane, where Lgr proteins are

not expressed, it would be unlikely to be able to perform its function as an agonist of the invention. Without this knowledge, the skilled person would not have known how to combine tissue-specific targeting with Lgr targeting and thus to generate the multi-targeting agonists as described herein. Therefore, in some embodiments the further target polypeptide or other target molecule is expressed basolaterally. In a further embodiment, the further target polypeptide is not expressed apically. Examples of basolaterally expressed targets include but are not limited to Epcam, A33 and L-cadherin.

The present inventors have surprisingly demonstrated that a Furin domain fragment (which mimics the activity of Rspondin binding to Lgr) linked to an anti-Epcam antibody can act as a substitute for Rspondin, even at 50x lower concentrations than Rspondin (see Example 4 and Figure 24).

In some embodiments, the further target polypeptide or other target molecule is a marker that is specifically expressed in Lgr5+ cells. The isolation of Lgr5+ stem cells is described for the first time in WO2009/022907. Lgr5+ stem cells differ from all previously described stem cells in that they are not quiescent, they have high levels of telomerase, they produce their own niche and they can survive more than 1000 divisions in stem cell culture medium (see WO2010/090513). Therefore, these cells differ from every other population of stem cells previously described or isolated. It is envisaged that knowledge of Lgr5+ stem cell markers can be combined with the surprising finding that Rspondin activates Wnt signalling through the Lgr-LRP-Frz complex, to generate multi-targeting compounds that have high avidity for Lgr5+ stem cells in particular target tissues of interest. In some embodiments, a multi-targeting compound of the invention, may target an Lgr5+ stem cell marker and Lgr5 and/or Lgr4 and/or Lgr6. In a preferred embodiment, a multi-targeting compound that targets an Lgr5+ stem cell marker will also target Lgr5, optionally without also targeting Lgr4 and/or Lgr6. In other embodiments, the multi-targeting compound that targets an Lgr5+ stem cell marker, and optionally targets Lgr5, will also target Lgr4 and/or Lgr6.

In some embodiments the invention provides a multi-targeting compound, comprising at least one portion with Rspondin-mimicking activity, and at least one other portion that binds to a further target polypeptide or other target molecule.

In some embodiments of the invention, wherein the agonist is a multi-targeting compound, the agonist of the invention comprises an Rspondin protein. By this it is meant that in some embodiments, the at least one portion with Rspondin-mimicking activity comprises an Rspondin protein. The Rspondin protein according to the invention may be Rspondin 1, Rspondin 2, Rspondin 3 or Rspondin 4. In other embodiments of the invention, the multi-targeting compound comprises an Rspondin variant, derivative or fragment as described herein. In some embodiments, the agonist is an Rspondin derivative that is specific to only Lgr4, Lgr5 or Lgr6. For example the agonist in some embodiments the agonist is an Rspondin derivative specific to only Lgr5, for example by mutation of one or more amino acid residues involved in binding to the Lgr4, Lgr5 and Lgr6 proteins. In other embodiments of the invention, the multi-targeting compound comprises a small-molecule agonist of the invention or any other agonist of the invention described herein.

The multi-targeting compound retains useful functionality in terms of enhancing Wnt signalling by mimicking the activity of Rspondin binding to an Lgr protein.

By useful functionality, it is meant that the multi-targeting agonist of the invention enhances β-catenin signalling in a cell, group of cells or a tissue by an amount that is useful for the intended purpose of the agonist, for example, by at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 300%, at least 400% or at least 500% compared to the β-catenin signalling induced by a neutral substance or negative control as measured in an assay described herein, for example as measured in the TOPFlash assay.

In some embodiments, a multi-targeting agonist of the invention enhances β-catenin signalling in a cell, group of cells or a tissue by an amount that is useful for the intended purpose of the agonist, for example, by at least 10x, at least 20x, at least 50x, at least

100x, at least 200x, at least 300x, at least 400x, at least 500x, at least 1000x, at least 2000x, at least 5000x, or at least 10,000x compared to the β-catenin signalling induced by Rspondin as measured in an assay described herein, for example as measured in the TOPFlash assay.

In some embodiments, the Rspondin is Rspondin 4. Rspondin 4 is a non-essential protein in mice and humans. Therefore, it is expected to result in fewer negative side-effects when used in therapy.

In other embodiments, the at least one portion with Rspondin-mimicking activity is any agonist of the invention described herein.

In some embodiments the at least one other portion that binds to a further polypeptide or other target molecule is a polypeptide, a peptidomimetic, an antibody or a fragment thereof, an aptamer or a small molecule. For example, in some embodiments the antibody is an anti-Epcam antibody. In other embodiments the small molecule is coagulation factor IX or complement component C4. The anti-Epcam antibody targets epithelial cells (see Example 4). Coagulation factor IX and complement component C4 target the liver.

In some embodiments in which the agonist of the invention binds to a further target polypeptide or other target molecule, the agonist may be a conjugate or a fusion protein.

As used herein, the term "conjugate" refers to two or more molecules that have been covalently joined, optionally by a linking region. For example, in some embodiments, a conjugate is a first protein or non-protein moiety joined to a second protein or non-protein moiety by a linking region. For example, in some embodiments of the invention a multi-targeting agonist comprises or consists of two antibodies that have been covalently joined. A conjugate is not limited to a first and second moiety but in some embodiments may also have a third, fourth or more moieties joined by further linking regions. As described elsewhere in this application, examples of protein moieties include, but are not limited to: a polypeptide, a peptidomimetic or an antibody or

fragment thereof. Examples of non-protein moieties include, but are not limited to: an aptamer or a small-molecule.

Numerous types of linker can be used, and the linker will be selected to be appropriate according to the molecule types in the conjugate and on the desired properties of the linker (length, flexibility, resistance to protease activity and other similar characteristics). Such linkers may comprise nucleotides, polypeptides, or a suitable synthetic material. For example, a linker may be a flexible peptide linker (for example, see SEQ ID NO: 142). In certain embodiments, the linker may be a cleavable linker, allowing the parts of the conjugate to be separated from each other. In other embodiments, a peptide linker might be a helical linker. Various examples and kits for linking proteins and other molecules are well known in the art.

As used herein, the term "fusion protein" refers to a protein that comprises two or more polypeptides or proteins that have been joined at the DNA level by recombination and are expressed together as a single polypeptide. A fusion protein may also comprise a peptide linking region also encoded by the DNA and expressed together with the fusion protein. A peptide linker that is part of a fusion protein, may be designed to have particular characteristics such as flexibility, hydrophilicity, protease-resistance, cleavability etc. All these properties can be designed within the DNA sequence and methods for designing linkers are well known in the art.

Where the agonist of the invention binds to a further target polypeptide or other target molecule (i.e. where it is a multi-targeting compound), it may be a bispecific antibody. A wide variety of multispecific recombinant antibody formats are known in the art, such as tetravalent bispecific antibodies by fusion of, e.g., an IgG antibody format and single chain domains[65, 66, 67]. For example, TandAbs® (Affimed) are tetravalent bispecific antibody formats that have two binding sites for each antigen. They bind to target molecules on the surface of, for example, tumour cells and can activate immune effector cells like cytotoxic T-cells or natural killer (NK) cells. TandAbs® possess the same avidity and affinity for each target as an IgG. Similarly, BiTE's (Bispecific T-cell engager molecules) constitute a class of bispecific single-chain antibodies for the

polyclonal activation and redirection of cytotoxic T cells against pathogenic target cells. Several other new formats wherein the antibody core structure (IgA, IgD, IgE, IgG or IgM) is no longer retained such as dia-, tria- or tetrabodies, minibodies, several single chain formats (scFv, Bis-scFv), which are capable of binding two or, more antigens, have also been developed[68, 69, 70, 71]. All such formats use linkers either to fuse the antibody core (IgA, IgD, IgE, IgG or IgM) to a further binding protein (e.g. scFv) or to fuse e.g. two Fab fragments or scFvs[69].

In one embodiment, where the agonist of the invention binds to a further target polypeptide or other target molecule (i.e. where it is a multi-targeting compound), it comprises or consists of two or more antibodies. In a further embodiment, at least one antibody is an agonist according to the present invention and at least one antibody binds to a tissue-specific cell marker.

Exemplary multi-targeting agonists

In one embodiment, the multi-targeting compound is an agonist of the invention linked to an antibody that binds to a tissue-specific marker.

In one embodiment, the multi-targeting compound is Rspondin 1-4 linked to an antibody that binds a tissue-specific marker.

In one embodiment, the multi-targeting compound is an Rspondin fragment, for example a Furin domain fragment, linked to an antibody that binds a tissue-specific marker.

In one embodiment the Rspondin fragment is represented by the sequence of amino acids recited in SEQ ID NO: 139 or SEQ ID NO: 140, SEQ ID NO: 141 or SEQ ID NO: 143.

In one embodiment, the multi-targeting compound comprises or consists of 1D9 linked to an antibody that binds a tissue-specific marker.

In one embodiment, the multi-targeting compound is a bispecific antibody which has a first portion with agonistic activity according to the invention i.e. a first portion that

mimics the activity of Rspondin binding to an Lgr protein, and a second portion that binds to a tissue-specific marker.

In a further embodiment the tissue-specific marker is selected from the list comprising: Epcam, A33, L-cadherin, CA19, dipeptidyl peptidase V, L-SIGN for endothelial cells of the liver and lymph nodes, CD-26 (also known as DPPIV), and Integrin a6b1.

Antagonists

Alternatively, the invention provides a bispecific compound, optionally a bi-specific antibody, which binds to both Lgr4 and Lgr5 or Lgr4 and Lgr6 or Lgr5 and Lgr6 and inhibits Wnt/β-catenin signalling. Similarly, the invention also provides mixtures of compounds that inhibit Rspondin binding to Lgr4 and Lgr5 or Lgr4 and Lgr6 or Lgr5 and Lgr6, optionally mixtures comprising anti-Lgr4 and anti-Lgr5 or anti-Lgr4 and anti-Lgr6 or anti-Lgr5 and anti-Lgr6 antibodies that inhibit Wnt/β-catenin signalling.

Antibodies that are antagonists of Lgr4 or Lgr5 and/or Lgr6 are known in the art (for example, see WO 2010016766), and can be easily generated by methods well-known in the art. For example, the inventors have demonstrated that the following anti-Lgr5 antibodies are antagonists of the Wnt pathway: 6C10, 2B6, 3B9, 8F2 and 9G5 (see Figure 28A). The inventors have demonstrated that the following anti-Lgr4 antibodies are antagonists of the Wnt pathway: 5C4, 4D4, 7F8, 4B4, 3C6, 3A11, 9B3, 2H10, 6C7, 8H9, 9B8 (see Figure 28B).

The inventors have discovered that antagonistic antibodies often bind to the N-terminal domain and/or one or more LRR of the Lgr protein, whereas examples of agonistic antibodies have been seen to target the CRL region (see Figure 4C in combination with Figure 28A and table 4). This information may be useful for the generation and/or design of new antagonists (and agonists) of Wnt/β-catenin signalling, including antagonistic antibodies (see the section "Methods for identifying further agonists"). In some embodiments, therefore, the anti-Lgr antibody that inhibits Wnt/β-catenin signalling, binds to the N-terminal domain and/or one or more LRR.

Antagonistic antibodies can be linked together by methods well-known in the art, and as described herein, to form bispecific or multi-targeting antagonists. Alternatively, they can be administered in combination. Where the antibodies are administered in combination they may be administered simultaneously, sequentially, or separately in any order.

Compounds which inhibit Wnt/β-catenin signalling are generally considered to show promise as therapeutics for cancer treatment. In WO 2009/005809 an Lgr5 antibody is described for possible treatment of cancer. However, in tissues where Lgr5 is always accompanied by Lgr4 expression, inhibition of Lgr5 alone would be insufficient for cancer treatment because, as is shown for the first time in the present application (see example 1), the inhibited Lgr5 is redundant and so Rspondin-mediated signalling could still occur through Lgr4. Therefore, a compound, or a mixture of compounds, that targets both Lgr4 and Lgr5 in these tissues would be clearly advantageous over a compound only targeting Lgr5, for effectively blocking Wnt/β-catenin signalling and reducing cellular proliferation for the treatment of cancer. The same reasoning also applies for Lgr6, which is also often expressed in combination with Lgr4.

In some embodiments, one or more of the compounds that inhibit Rspondin binding to Lrg4 or Lgr5 or Lgr6 (i.e. which bind to and "inhibit" the activation of Lgr4, Lgr5 and/or Lgr6) are selected from the group consisting of: a small-molecule, a polypeptide, an antibody or fragment thereof, a biological antagonist, an aptamer and an antisense oligonucleotide. As used herein, a biological antagonist of Lgr4 or Lgr5 or Lgr6 refers to a compound that inhibits Lgr4 or Lgr5 or Lgr6 that occurs naturally in biological cells, for example, a biological antagonist may be a protein or a siRNA.

Accordingly, the invention provides a method of inhibiting β-catenin signalling comprising administering a bispecific antibody or a mixture of compounds that inhibit Lgr4 and Lgr5 or Lgr4 and Lgr6 or Lgr5 and Lgr6. The method may be conducted *in vitro, in vivo* or *ex vivo*. Preferably, the method is conducted *in vivo* and is administered to a patient to treat cancer.

Also provided is a method of inhibiting β-catenin signalling comprising administering a compound that inhibits Rspondin binding to Lgr4 in combination with a compound that inhibits Rspondin binding to Lgr5, or a compound that inhibits Rspondin binding to Lgr4 in combination with a compound that inhibits Rspondin binding to Lgr6, or a compound that inhibits Rspondin binding to Lgr5 in combination with a compound that inhibits Rspondin binding to Lgr6, wherein said compounds are administered simultaneously, sequentially, or separately in any order.

Thus the invention provides a method for treating disorders associated with abnormal tissue growth such as cancer, comprising administering one, two or all three of a compound that inhibits Lgr5, a compound that inhibits Lgr4 and a compound that inhibits Lgr6 to a patient, wherein the compounds are administered simultaneously, sequentially or separately in any order.

The invention also provides a compound that inhibits Lgr4 for use in treating a patient who has previously been treated with a compound that inhibits Lgr5. Conversely, the invention also provides a compound that inhibits Lgr5 for use in treating a patient who has previously been treated with a compound that inhibits Lgr4.

The invention also provides a compound that inhibits Lgr4 for use in treating a patient who has previously been treated with a compound that inhibits Lgr6. Conversely, the invention also provides a compound that inhibits Lgr6 for use in treating a patient who has previously been treated with a compound that inhibits Lgr4.

The invention also provides a compound that inhibits Lgr6 for use in treating a patient who has previously been treated with a compound that inhibits Lgr5. Conversely, the invention also provides a compound that inhibits Lgr5 for use in treating a patient who has previously been treated with a compound that inhibits Lgr6.

In some embodiments, the invention also provides a kit comprising a compound that inhibits Lgr5 and a compound that inhibits Lgr4. In some embodiments, the invention also provides a kit comprising a compound that inhibits Lgr5 and a compound that inhibits Lgr6. In some embodiments, the invention also provides a kit comprising a

compound that inhibits Lgr4 and a compound that inhibits Lgr6. In some embodiments, the invention also provides a kit comprising a compound that inhibits Lgr4 and a compound that inhibits Lgr6 and a compound that inhibits Lgr5. The compound that inhibits Lgr5 and/or the compound that inhibits Lgr4 and/or the compound that inhibits Lgr6 may be for simultaneous, sequential or separate administration. In a further embodiment, the kit of the invention is for use in treating a patient. In a further embodiment, the kit of the invention is for use in accordance with any of the methods of the invention, wherein the methods comprise inhibiting β-catenin signalling.

Cancers that would benefit from combined Lgr4/5 modulation provided by mixtures or bispecifics of the present invention, would be cancers that lack mutationally activated Wnt signalling, i.e. cancers that have normal Wnt signaling. Examples of such cancers include: breast cancer, ovarian cancer, lung cancer, prostate cancer, or skeletal cancer (e.g. osteosarcoma). It is believed that in cancers wherein the intracellular Wnt signalling is disturbed (mutationally activated, for instance APC mutations in colon cancer) blocking Lgr4 or Lgr5 would have no effect.

Although bispecific compounds have been described in detail, it is to be understood that multi-targeting antagonists would also be possible. For example, in one embodiment the invention provides a multi-targeting antagonist that inhibits activation of Lgr4, Lgr5 and Lgr6. In one embodiment, the multi-targeting antagonist may comprise of an anti-Lgr4 antibody, an anti-Lgr5 antibody and an anti-Lgr6 antibody. In some embodiments, the multi-targeting antagonists inhibit activation by preventing Rspondin, and optionally an Rspondin-mimicking agonist, from binding to Lgr4, Lgr5 and/or Lgr6, or alternatively from activating Lgr4, Lgr5 and/or Lgr6.

In a further embodiment the invention provides antagonists that block activation of the Wnt pathway by binding to the Rspondin binding site or the antibody 1D9 binding site. For example, the invention provides antagonists that block the activity of an agonist binding to the CRL region or to the LRRs of an Lgr protein.

Compositions

As discussed in more detail below, the agonists of the invention have widespread medical applications. The invention therefore further provides a pharmaceutical composition comprising one or more agonist according to the invention and a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers and excipients are known in the art and described, for instance, in "Remington; The Science and Practice of Pharmacy"[73].

The one or more agonists according to the invention may be any combination of agonists that mimic the effect of Rspondin binding to Lgr4, Lgr5 or Lgr6 (also referred to herein as Lgr4, Lgr5 and Lgr6 agonists), including for example small molecules, antibodies, multi-targeting compounds etc as described herein. A composition of the invention, for example, comprising an Lgr4 agonist of the invention and an Lgr5 agonist of the invention might be expected to be more effective at enhancing Wnt signalling in certain tissues, compared to a composition only containing an Lgr5 agonist. Other combinations of agonists may have other beneficial effects according to the particular tissue-specific expression patterns of Lgr4, Lgr5 and Lgr6, outlined elsewhere in the application.

In one embodiment, the compositions comprising one or more agonists of the invention may comprise one or more antibody for Lgr4, Lgr5 or Lgr6 according to the invention, or any combination of the above. For example, the composition may comprise an agonistic anti-Lgr4 antibody and an agonistic anti-Lgr5 antibody. Alternatively, the composition may comprise an agonistic anti-Lgr4 antibody and an agonistic anti-Lgr6 antibody. Alternatively, the composition may comprise an agonistic anti-Lgr5 antibody and an agonistic anti-Lgr6 antibody. Alternatively, the composition may comprise an agonistic anti-Lgr4 antibody, an agonistic anti-Lgr5 antibody and an agonistic anti-Lgr5 antibody.

In one embodiment, the compositions comprising one or more agonists of the invention, also comprise Wnt and/or a suitable Wnt substitute that binds and activates the Wnt pathway via Frizzled and LRP.

The compositions comprising one or more agonists of the invention may be used to treat a range of disorders associated with tissue loss or damage due to aging or pathological conditions. The pharmaceutical compositions of the invention may therefore include other drugs known to be useful in the treatment of these conditions. The pharmaceutical composition further comprises an effective amount of at least one compound or protein selected from at least one of: an anti-infective drug, a cardiovascular (CV) system drug, a central nervous system (CNS) drug, an autonomic nervous system (ANS) drug, a respiratory tract drug, a gastrointestinal (GI) tract drug, a hormonal drug, a drug for fluid or electrolyte balance, a hematologic drug, an antineoplastic, an immunomodulation drug, an ophthalmic, otic or nasal drug, a topical drug, or a nutritional drug.

The invention also provides a pharmaceutical composition comprising two or more antagonists according to the invention and a pharmaceutically acceptable carrier or excipient. The two or more antagonists may be any combination of compounds that inhibit binding of Rspondin to Lgr4, Lgr5 or Lgr6. In a preferred embodiment, the two or more antagonists inhibit binding of Rspondin to Lgr4 and Lgr5.

The compositions comprising one or more antagonists of the invention may be used to treat a range of disorders in which it is desirable to inhibit/antagonise Wnt signalling, for example disorders associated with abnormal tissue growth, such as cancer. The pharmaceutical compositions of the invention may therefore include other drugs known to be useful in the treatment of these conditions. The pharmaceutical composition further comprises an effective amount of at least one compound or protein selected from at least one of: an anti-infective drug, a cardiovascular (CV) system drug, a central nervous system (CNS) drug, an autonomic nervous system (ANS) drug, a respiratory tract drug, a gastrointestinal (GI) tract drug, a hormonal drug, a drug for fluid or electrolyte balance, a hematologic drug, an antineoplastic, an immunomodulation drug, an ophthalmic, otic or nasal drug, a topical drug, a nutritional drug or a cancer drug, such as Tamoxifen.

The compositions of the invention may be formulated so that it is suitable for administration by any administration routes known in the art, for example intravenous, subcutaneous, intramuscular administration, mucosal, intradermal, intracutaneous, oral,

and ocular. A pharmaceutical composition may be thus be in any form suitable for such administration, e.g. a tablet, infusion fluid, capsule, syrup, etc.

Method of Treatment

The present invention provides an agonist or a composition according to the invention for use in therapy. In some embodiments, the invention provides an agonist or a composition comprising one or more agonists according to the invention for use in treating diseased or damaged tissue, for use in tissue regeneration and for use in cell growth and proliferation, and/or for use in tissue engineering. In particular, the present invention provides an agonist or a composition comprising one or more agonists according to the invention for use in treating tissue loss or damage due to aging or pathological conditions.

In some embodiments of the invention, the agonist of composition for use in therapy comprises or consists of antibody 1D9 and/or a multi-targeting compound comprising an anti-Epcam antibody linked to a Rspondin Furin domain fragment.

The invention also provides a method for treating tissue loss or damage due to aging or pathological conditions by administering an agonist or composition comprising one or more agonists of the invention to a patient. The patient may be any animal (e.g., a mammal), including, but not limited to, humans, non-human primates, rodents, and the like. Typically, the patient is human. The methods of treatment and medical uses of the agonists of the invention or compounds or compositions comprising agonists of the invention promote tissue regeneration. The term "tissue" refers to part of an organism consisting of a cell or an aggregate of cells, optionally having a similar structure, function and/or origin. Examples of tissues include but are not limited to: epithelial tissues, such as skin tissue, stomach lining, pancreatic lining, liver; connective tissues, such as inner layers of skin, tendons, ligaments, cartilage, bone, fat, hair, blood, muscle tissues; and nerve tissues, such as glial cells and neurons. The loss or damage can be anything which causes the cell number to diminish. For example, an accident, an autoimmune disorder, a therapeutic side-effect or a disease state could constitute trauma.

Specific examples of conditions which may cause cell number to diminish include, but are not limited to: radiation/chemotherapy, mucositis, IBD, short bowel syndrome, hereditary bowel disorders, celiac disease, metabolic diseases, hereditary syndromes, (viral) infections (hepB/C), toxic states, alcoholic liver, fatty liver, cirrhosis, infections, pernicious anemia, ulceration, diabetes, destruction of islet cells, loss of bone mass (osteoporosis), loss of functional skin, loss of hair, loss of functional lung tissue, loss of kidney tissue (for instance acute tubulus necrosis), loss of sensory cells in the inner ear. Tissue regeneration increases the cell number within the tissue and preferably enables connections between cells of the tissue to be re-established, and more preferably the functionality of the tissue to be regained.

Other conditions that may be treated with the agonists or compositions comprising one or more agonists of the invention include but are not limited to: joint disorders, osteoporosis and related bone diseases, baldness, graft-versus-host disease.

Agonists or compositions comprising one or more agonists of the invention, in particular agonists that bind and activate Lgr6, may also be used for wound healing and generation of smooth muscle tissues in many organs (e.g. airways, large arteries, uterus).In some embodiments, the invention provides methods of treatment and medical uses, as described previously, wherein two or more agonist of the invention or compounds or compositions comprising agonists of the invention, are administered to an animal or patient simultaneously, sequentially, or separately.

In some embodiments, the invention provides methods of treatment and medical uses, as described previously, wherein one or more agonist of the invention or compounds or compositions comprising agonists of the invention, is administered to an animal or patient in combination with one or more further compound or drug, and wherein said agonist of the invention or compounds or compositions comprising agonists of the invention and said further compound or drug are administered simultaneously, sequentially, or separately.

Examples of further compounds or drugs include but are not limited to: an anti-infective drug, a cardiovascular (CV) system drug, a central nervous system (CNS) drug, an

autonomic nervous system (ANS) drug, a respiratory tract drug, a gastrointestinal (GI) tract drug, a hormonal drug, a drug for fluid or electrolyte balance, a hematologic drug, an antineoplastic, an immunomodulation drug, an ophthalmic, otic or nasal drug, a topical drug, or a nutritional drug.

In one embodiment, the one or more further compound or drug comprises or consists of Wnt and/or a suitable Wnt substitute that binds and activates the Wnt pathway via Frizzled and LRP.

Methods for enhancing cell growth/proliferation

The agonists of the invention also have widespread applications in non-therapeutic methods, for example *in vitro* research methods.

The invention thus provides a method for tissue regeneration of damaged tissue, such as the tissues discussed in the section of medical uses above, comprising administering an agonist of the invention. The agonist may be administered directly to the cells *in vivo*, administered to the patient orally, intravenously, or by other methods known in the art, or administered to *ex vivo* cells. In some embodiments where the agonist of the invention is administered to *ex vivo* cells, these cells may be transplanted into a patient before, after or during administration of the agonist of the invention.

The invention also provides a method for enhancing the proliferation of cells comprising supplying the cells with an agonist of the invention.

These methods may be carried out *in vivo*, *ex vivo* or *in vitro*.

Wnt agonists are key components of stem cell culture media. For example, the stem cell culture media as described in WO2010/090513, WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 2011;141:1762–1772) and Sato et al., 2009 (Nature 459, 262-5) comprise Rspondin. The agonists of the invention that mimic Rspondin are thus suitable alternatives to Rspondin for use in these stem cell culture media. No alternatives to Rspondin in this context are already known.

The inventors have shown that antibody 1D9 can act as a substitute for Rspondin for culturing cells (see Example 3 and Figure 23). The inventors have also shown that a multi-targeting compound comprising an anti-Epcam antibody linked to a Rspondin Furin domain fragment can act as a substitute for Rspondin for culturing cells. Furthermore, they have shown that this multi-targeting compound has a similar effect to Rspondin on cell growth even at 50x lower concentrations (see Example 4 and Figure 24).

Accordingly, in one embodiment, the invention provides a method for enhancing the proliferation of stem cells comprising supplying stem cells with an agonist of the invention. In one embodiment, the invention provides a cell culture medium comprising one or more agonist of the invention. In some embodiments, the cell culture medium may be any cell culture medium already known in the art that normally comprises Rspondin, but wherein the Rspondin is replaced (wholly or partially) or supplemented by an agonist of the invention which mimics the activity of an Rspondin protein binding to an Lgr protein. For example, the culture medium may be as described in as described in WO2010/090513, WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 2011;141:1762–1772) and Sato et al., 2009 (Nature 459, 262-5), which are hereby incorporated by reference in their entirety.

For example, in one embodiment the invention provides a method for enhancing the proliferation of stem cells comprising supplying stem cells with antibody 1D9. In one embodiment, the invention provides a cell culture medium comprising antibody 1D9.

In another embodiment the invention provides a method for enhancing the proliferation of stem cells comprising supplying stem cells with a multi-targeting compound comprising an anti-Epcam antibody linked to a Rspondin Furin domain fragment. In one embodiment, the invention provides a cell culture medium comprising a multi-targeting compound comprising an anti-Epcam antibody linked to a Rspondin Furin domain fragment.

In a further embodiment the cell culture medium comprising one or more agonists of the invention, further comprises a receptor tyrosine kinase ligand (for example a growth

factor, such as EGF) and a BMP inhibitor (for example Noggin). Such cell culture media are appropriate for growing organoids comprising Lgr5+ cells.

In a further embodiment, the cell culture medium comprising one or more agonists of the invention also comprises Wnt and/or a suitable Wnt substitute that binds and activates the Wnt pathway via Frizzled and/or LRP.

Stem cell culture media often comprise additional growth factors. This method may thus additionally comprise supplying the stem cells with a growth factor.

Growth factors commonly used in cell culture medium include epidermal growth factor (EGF, (Peprotech), Transforming Growth Factor-alpha (TGF-alpha, Peprotech), basic Fibroblast Growth Factor (bFGF, Peprotech), brain-derived neurotrophic factor (BDNF, R&D Systems), Human Growth Factor (HGF) and Keratinocyte Growth Factor (KGF, Peprotech, also known as FGF7). EGF is a potent mitogenic factor for a variety of cultured ectodermal and mesodermal cells and has a profound effect on the differentiation of specific cells in vivo and in vitro and of some fibroblasts in cell culture. The EGF precursor exists as a membrane-bound molecule which is proteolytically cleaved to generate the 53-amino acid peptide hormone that stimulates cells.

EGF or other mitogenic growth factors may thus be supplied to the stem cells. During culturing of stem cells, the mitogenic growth factor may be added to the culture medium every second day, while the culture medium is refreshed preferably every fourth day. In general, a mitogenic factor is selected from the groups consisting of: i) EGF, TGF-α and KGF, ii) EGF, TGF-α and FGF7; iii) EGF, TGF-α and FGF; iv) EGF and KGF; v) EGF and FGF7; vi) EGF and a FGF; vii) TGF-α and KGF; viii) TGF-α and FGF7; ix) or from TGF-α and a FGF.

These methods of enhancing proliferation of stem cells can be used to grow new organoids and tissues from stem cells, as for example described in WO2010/090513 WO2012/014076, Sato et al., 2011 (GASTROENTEROLOGY 2011;141:1762–1772) and Sato et al., 2009 (Nature 459, 262-5).

In some embodiments the invention provides organoids obtained using the methods and/or media of the invention.

Methods for identifying further agonists

The identification of the mechanism by which Rspondin induces activation of the Wnt signalling pathway will also enable the identification of further agonists that mimic Rspondin activity. The identification of 1D9 as an agonist of Lgr5, and knowledge of the region where 1D9 binds on Lgr5 will also help identify and engineer new agonists of Lgr5, Lgr6 and Lgr4.

Based on the molecular structures of the variable regions of an anti- Lgr4, Lgr5 and/or Lgr6 antibody of the invention, molecular modelling and rational molecular design can be used to generate and to screen molecules which mimic the molecular structures of the binding region of the antibodies and thus mimic the activity of Rspondin binding to the Lgr proteins. These small molecules may be peptides, aptamers, peptidomimetics, oligonucleotides, antibodies or other organic compounds. Alternatively, one could use large-scale screening procedures commonly used in the field to isolate suitable molecules from libraries of compounds.

Common mutational techniques may be used to modify antibody 1D9 to generate new agonists with improved properties (e.g. in terms of increased beiding affinity to an Lgr protein, increased activity in terms of activating the Wnt pathway, reduced toxicity, greater solubility etc.). In addition, now that it is known that antibody 1D9 is an agonist and binds to the hinge (CRL) region of Lgr5 (see sections "Binding of the agonist to Lgr" and "Exemplary agonists" above), it is also possible to isolate the hinge region of Lgr4, Lgr5 or Lgr6 (or similar fragments comprising or consisting of the 1D9 binding sites) and to generate antibodies that bind to this specific region by methods well known in the art. The isolate hinge region of Lgr4, Lgr5 or Lgr6 (or similar fragments comprising or consisting of the 1D9 binding site) could also be used to screen for small molecules that bind this specific region by methods well known in the art. It would be expected that such antibodies or small molecules that bind this specific region would be

more likely to behave as agonists and may also be more likely to be specific for Lgr5 (or Lgr4 or Lgr6).

Furthermore, now that the interaction between Lgr and Rspondin is known, and now that the agonistic activity of antibody 1D9 on Lgr5 is known, crystal structures, NMR or other structural information about the interacting amino acids that are involved in the Lgr-Rpsondin interaction and/or the Lgr-antibody (e.g. Lrg5-1D9) interaction, and/or site specific mutagenesis could be used to identify key interacting amino acids.

For example, an Rspondin derivative or fragment may be engineered to have increased specificity for Lgr5 compared to Lgr4 or Lgr6. Alternatively, an Rspondin derivative or fragment may bind and activate only Lgr5 and not Lgr4 or Lgr6. In an alternative embodiment, the Rspondin derivative or fragment is specific for, or has greater specificity for Lgr4 (relative to Lgr5 and/or Lgr6) or Lgr6 (relative to Lgr4 and/or Lgr5). This would be advantageous for use in therapy, for example, because wild-type Rspondin binds to Lgr4, Lgr5 and Lgr6 and stimulates growth, via the Wnt pathway, in all proliferating cells. An Rspondin derivative or fragment that has increased specificity for Lgr5, would preferentially stimulate growth of Lgr5-positive stem cells, not in all dividing cells. Therefore, in situations where wild-type Rspondin causes too much regrowth, an Rpsondin derivative or fragment with specificity for Lgr5 would be more appropriate for regenerative treatments.

The invention thus provides a method for identifying an agonist of the Wnt pathway, said method comprising:

a) Isolating a peptide fragment of an Lgr protein comprising or consisting of the region of Lgr that binds 1D9 and/or Rspondin;
b) Raising an antibody against said peptide fragment;
c) Testing said antibody for agonistic activity.

In an alternative embodiment, invention provides a method for identifying an antagonist of the Wnt pathway, said method comprising:

a) Isolating a peptide fragment of an Lgr protein comprising or consisting of the region of Lgr that binds 1D9 and/or Rspondin;
b) Raising an antibody against said peptide fragment;
c) Testing said antibody for antagonistic activity.

The invention also thus provides a method for identifying an agonist of the Wnt pathway, said method comprising:

a) contacting a complex comprising at least one Lgr protein, at least one Frizzled receptor and at least one LRP protein with a candidate compound in the presence of a Wnt protein; and

b) determining the level of Wnt/beta catenin signalling

wherein an increase in the level of Wnt/beta catenin indicates that the candidate compound is an agonist of the Wnt pathway.

The method may be a cell-based assay conducted *in vitro*.

The Lgr protein is at least one of Lgr4, Lgr5 or Lgr6. The Frz is at least one of Frizzled 1-10, typically at least one of Frz5, Frz6 and Frz7. The LRP co-receptor in the complex may be any LRP protein family member. The complex much thus comprise at least one of LRP5 or LRP6. The Wnt protein may be any Wnt family member.

The complex may comprise, for example, Wnt and Frz5-LRP6, Frz6-LRP6, Frz7-LRP6 and other combinations of the proteins described above. In particular, the Lgr protein may be Lgr4, Lgr5 and/or Lgr6 in a complex comprising Frz5, Frz6, and/or Frz7, and LRP5 and/ or LRP6, as well as Wnt.

In some embodiments the Lgr protein in the method for identifying an agonist of the Wnt pathway may be a fragment of an Lgr protein. For example, in some embodiments,

the fragment of Lgr comprises or consists of the hinge region (or CRL region) of Lgr4, Lgr5 or Lgr6.

An agonist in this context is a compound that mimics the activity of Rspondin, for example by binding an Lgr protein selected from Lgr4, Lgr5 or Lgr6, on a cell and initiates a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand Rspondin i.e. the agonist enhances Wnt signalling in the cell.

An increase in the level of Wnt/β-catenin signalling according to this screening method may be detected by identification of an increase in any of the following responses indicative of Wnt signalling: β-catenin stability, transcription of TCF-induced genes, LRP phosphorylation, axin translocation from cytoplasm to cell membrane and binding to LRP. Methods for determining the level of Wnt/beta-catenin signalling of candidate compounds are discussed in detail above and any of these assays may be used in the method of this aspect of the invention to assess whether a candidate compound is a Wnt agonist.

The candidate compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries, peptoid libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are preferred for use with peptides, while the other four approaches are applicable to peptides, non-peptide oligomers or small molecules. Examples of methods for the synthesis of molecular libraries can be found in the art[73, 74, 75, 76, 77, 78, 79].

Libraries of compounds can be presented in solution[80], or on beads[81], chips[82], bacteria or spores[83], plasmids[84] or on phage[85, 86, 87, 88].

The candidate compounds may also be antibodies, such as the antibodies described above

The candidate compounds may be selected by conducting a preliminary step of screening for compounds that bind to the Lgr protein and in particular that bind to the agonistic epitopes of the Lgr proteins discussed above.

Preferably, the method of screening for compounds that bind Lgr includes: (a) mixing Lgr and one or more candidate compounds; (b) incubating the mixture to allow Lgr and the candidate compound(s) to interact; and (c) assessing whether the candidate compound binds to the Lgr protein.

This method may be conducted when the Frz receptors and LRP co-receptors described above are also present, and in particular when the Lgr protein is bound in a complex with Frz receptors and LRP co-receptors. This complex would mimic the "Wnt receptor complex" that comprises Lgr proteins, Frz receptors and LRP co-receptors and that has now been identified by the inventors

Various assays for assessing binding of candidate compounds to Lgr are well known in the art, such as BIA, SPR, ELISA, FRET, Kinexa, FACS, and mass spectrometry.

Biomolecular interaction analysis (also known as BIA e.g., BIAcore 3000, ProteOn XPR36) utilises surface plasmon resonance to detect biospecific interactions in real time, without the need to label any of the interactants[89]. Changes in the mass at the binding surface indicate a binding event and are measurable in terms of alterations of the refractive index of light near the binding surface. BIA may be used to characterise any kind of biomolecular interaction, including interactions between DNA-DNA, DNA-protein, lipid-protein and hybrid systems of biomolecules and non-biological surfaces can be investigated. Biomolecular Interaction Analysis can be used, for example, to identify the binding of two or more interactants to each other, to determine the affinity of the interactions, to measure the actual association and dissociation rates, and to determine whether two compounds bind competitively or cross-block in a competition binding assay.

The terms "cross-block", "cross-blocked" and "cross-blocking" are used interchangeably herein to mean the ability of an antibody or other binding agent to interfere with the

binding of other antibodies or binding agents to a certain epitope in a standard competitive binding assay. The ability or extent to which an antibody or other binding agent is able to interfere with the binding of another antibody or binding molecule to an epitope, and therefore whether it can be said to cross-block according to the invention, can be determined using standard competition binding assays.

The sandwich ELISA assay is an enzyme-linked immunosorbent assay which involves the immobilisation of a candidate compound on a solid support, such as a polystyrene microtitre plate. The antigen (e.g. an Lgr protein) is added and allowed to interact with the candidate compound. After washing with mild detergent solution to remove any protein that is not specifically bound, a detection antibody which is specific for the antigen is added. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. After another wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. A number of variations of the ELISA assay exist and are well known to those skilled in the art. For example, the competition ELISA assay relies on the ability of a labeled analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance.

The KinExA method is in particular useful for determining binding kinetics. The KinExA method measures the concentration of receptor (e.g. Lgr) molecule in a mixture of receptor, ligand (e.g. candidate compound), and ligand-receptor complex. The concentration of uncomplexed receptor is measured by exposing the solution phase mixture to solid phase immobilized ligand for a very brief period of time. The "contact time" between the solution phase mixture and the solid phase immobilized ligand is kept short enough that dissociation of ligand-receptor complex is insignificant. When the possibility of significant dissociation of ligand-receptor complex is kinetically excluded, only uncomplexed ("free") receptor can bind to the solid phase. The amount of free receptor that binds to the solid phase (measured by fluorescence emission from a

secondary label) is directly proportional to the concentration of free receptor in the solution phase sample.

The interaction between two molecules can also be detected using Förster resonance energy transfer (FRET)[90, 91]. FRET is the phenomenon wherein a donor fluorophore, initially in its electronic excited state, may transfer energy to an acceptor fluorophore in close proximity through nonradiative dipole–dipole coupling. The first compound of interest (e.g. the candidate compound) is labeled with a donor and the second compound of interest (e.g. the Lgr protein) is labeled with an acceptor. When they are dissociated, the donor emission is detected upon the donor excitation. However, when the donor and acceptor are mixed and come into close proximity (1-10 nm) due to the interaction of the two molecules, the acceptor emission is predominantly observed because of the intermolecular FRET from the donor to the acceptor. An FRET binding event can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorometer).

Affinity purification mass spectrometry is also a well-known approach for the characterisation of protein complexes (reviewed by Bauer, A. & Kuster, B.[92])[93]. Protein complexes may first be isolated by a multitude of different techniques ranging from size exclusion or ion exchange chromatography to different varieties of affinity chromatography. For example, the protein complexes may be captured on affinity columns by antibodies specific for the protein of interest, or by more generic antibodies that capture fusion proteins containing epitope tags, such as Myc, HA, Flag, KT3. Tandem affinity purification employs a similar technique, except that the protein of interest is given two tags, and two affinity steps are used which reduces the amount of unspecific protein binding. Once the protein complex has been isolated, the component parts are then identified. Characterisation of the proteins in the complex may be carried out by mass spectrometry. A preferred approach is tandem affinity purification followed by mass spectrometry (see example 1). A number of mass spectrometry approaches may be employed and are well known and reviewed in the art[94, 95].

Once a candidate compound has been identified *in vitro* as a compound that is an agonist of the Wnt pathway, it may be desirable to perform further experiments to confirm the *in vivo* function of the compound.

Any of the above methods may therefore comprise the further steps of administering to a non-human animal a candidate compound and assessing its effect on enhancing cellular proliferation, for example, in terms of tissue regeneration.

The invention also provides a method of assessing the *in vivo* effect on cellular proliferation of a compound obtained or obtainable by any of the methods described above, comprising administering the compound to a human or animal and assessing the effect on cellular proliferation, for example, in terms of tissue regeneration.

General

"GI" numbering is used above. A GI number, or "GenInfo Identifier", is a series of digits assigned consecutively to each sequence record processed by NCBI when sequences are added to its databases. The GI number bears no resemblance to the accession number of the sequence record. When a sequence is updated (*e.g.* for correction, or to add more annotation or information) then it receives a new GI number. Thus the sequence associated with a given GI number is never changed.

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

The term "about" in relation to a numerical value *x* is optional and means, for example, $x \pm 10\%$.

Unless specifically stated, a process comprising a step of mixing two or more components does not require any specific order of mixing. Thus components can be mixed in any order. Where there are three components then two components can be combined with each other, and then the combination may be combined with the third component, *etc.*

Various aspects and embodiments of the invention are described below in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

DESCRIPTION OF THE DRAWINGS

Figure 1: Conditional deletion of *Lgr4* and *Lgr5* in mouse small intestine.
Fig 1A: Top: Expression of Lgr5-LacZ is specific to small stem cells located between Paneth cells at crypt bottoms. Bottom: Expression of Lgr4-lacZ[96] occurs throughout intestinal crypts (note lower magnification of right panel).
Fig 1B: Design of *Lgr5*^{fl} allele. Black triangles indicate the inserted *loxp* sites. *PMCNeo* was removed by cre-mediated deletion in the germline.
Fig 1C: Adult mice homozygous for *Lgr4*^{fl} and/or *Lgr5*^{fl}, and carrying the *Ah-Cre* transgene, were analyzed five days post-β-naphtoflavone-induced deletion[97]. Left/middle panels: Proliferative cells are visualized by Ki67 staining. Right panels: Stem cells are visualized by OlfM4 *in situ* hybridization. Deletion of *Lgr5* has no obvious effect. Deletion of *Lgr4* has significant deleterious effects on crypt stem cells and proliferative progenitors as evidenced by loss of Ki67 crypts and Olfm4 signals. Upon *Lgr4/5* double deletion, >80% of crypts disappear as visualized by loss of Ki67^{+} compartments and OlfM4 expression.

Figure 2: Wnt target genes are downstream of Lgr4/5
Concomitant deletion of *Lgr4* and *Lgr5* in *Lgr4*$^{fl/fl}$-*Lgr5*$^{fl/fl}$ mice resulted in the significant downregulation of 307 unique genes one day post deletion (Table 2). The intestinal Wnt signature can be revealed by two opposing experiments: deletion of *Apc in vivo* results in

upregulation of a Wnt target signature[98], whereas Rspo-1 withdrawal from intestinal organoids[99] results in the immediate downregulation of Wnt target genes.

Fig2A: Heatmap of the log2 ratio of *Apc*$^{fl/fl}$ mice vs. wt mice for the 307 Lgr4/5 genes 3 days after deletion of *Apc* (ratios taken from Van Es et al. 2005[100]).

Fig2B: Heatmap of the log2 ratio of intestinal organoids 1 day after Rspo-1 withdrawal (-Rspo1) versus control organoids (+Rspo1) for the 307 *Lgr4/5* gene set.

Fig2C: Gene Set Enrichment Analysis (GSEA). Genes are ranked according to their differential expression between *Apc*-deleted and wt mice (data from Sansom, O.J. et al. 2007[98]). Black bars beneath the graph depict the rank positions of the 306 genes from the *Lgr4/5* gene set. A highly significant enrichment of the 306 *Lgr4/5* genes was detected towards the gene set upregulated 3 days after *Apc* deletion *in vivo*.

Fig2D: Genes are ranked according to their differential expression in intestinal organoids after Rspondin1 (Rspo-1) withdrawal versus control organoids. GSEA shows a highly significant enrichment of the 306 *Lgr4/5* genes towards the genes downregulated after Rspo-1 withdrawal. ES: enrichment score, NES normalized ES, FDR: false discovery rate.

Figure 3: Lgr4 and -5 proteins interact with Frizzled/Lrp complexes and Rspo-1 as assessed by mass spectrometry.

Left panel (Exps 1-7): HEK293T cells were transiently transfected, and LS174T cells were stably, transfected with the indicated tagged proteins. Right panel (Exps 8-9): HEK293T cells were incubated with the indicated tagged soluble proteins at 1 µg/ml. Sequential immunoprecipitation was performed by anti-FLAG and anti-HA antibodies and the associated complexes were analysed by mass spectrometry. Number of assigned spectra, number of unique peptides and the MASCOT Protein Score are given for selected identified proteins.

Figure 4: Direct physical interaction of Rspo-1 with Lgr4/5/6 exodomains

Figure 4A: Soluble Rspo-1 binds to Lgr4, Lgr5 and Lgr6. Top panel: HEK293T cells were transiently transfected ("tx") with FLAG/HA tagged versions of Lgr4, Lgr5 or Lgr6, and with tagged Frizzled5 (Frz5) or mock as control, incubated with a conditioned medium containing an Rspo1-Fc fusion protein at ~1 µg/ml where indicated. Cells were

washed, lysed and Rspo1-Fc was immunoprecipitated with proteinG beads. Western blotting for the FLAG tag revealed binding of soluble Rspo1-Fc to Lgr4, Lgr5 and Lgr6, but not to Frz5 or mock-transfected cells. Bottom panel: Western blotting of cell lysates for the FLAG tag reveals the presence of the transfected, tagged proteins.

Figure 4B: Rspo-1-Lgr5 interaction visualized by Surface Plasmon Resonance array imaging. Anti-FLAG antibody spotted on the sensor chip, readily captured Rspo-1-FH (left). The extracellular domain of Lgr5 was expressed as a human IgFc fusion protein (Lgr5-exo-Fc). After a wash, Lgr5-exo-Fc bound to Rspo-1-FH. After regeneration with low pH, a Noggin-Fc fusion protein served as negative control (right). Both Lgr5-exo-Fc and Noggin-Fc could be captured on goat anti-human IgG spotted as a control.

Figure 4C: Epitope mapping of anti-human Lgr5 antibodies. Ab1 recognizes the cystein-rich linker (CRL)-region in isolation. Mapping of all other epitopes was performed by human-mouse hybrid Lgr5 clones (white bars indicate regions of human origin), allowing mapping of the human-specific antibodies as indicated. LRR: leucine-rich repeat region.

Fig 4D: N-terminal-binding antibodies block Lgr5-exo-Fc/Rspo-1 interaction. The Lgr5 fusion protein was pre-incubated with the individual monoclonal antibodies followed by incubation with Rspo-1-FH bound to anti-FLAG beads. Blocking is visualized as loss of Lgr5-exo-Fc bound to the Rspo-1-coated beads by Western blotting for the Fc portion.

Figure 5: Lgr4 is essential for transmitting Rspo-1 signals but dispensable for transmitting Wnt3A signals.

Figure 5A: Downregulation of Lgr4 in HEK293T cells by transient transfection of three siRNAs (A, B, C) targeting the 3'UTR of Lgr4 or a scrambled siRNA (I). Expression assessed by Northern blotting three days post-transfection (top). Ethidiumbromide-stained gel shows equal loading as judged by ribosomal RNA bands (bottom).

Figure 5B: TOPflash Wnt reporter assays. HEK293T cells were transfected with three different siRNAs (A, B, C) targeting the 3'UTR of Lgr4, or a non-targeting control siRNA (I). Three days later, the cells were transfected with TOPflash Wnt reporters +/- 5

ng of the indicated human Lgr4, Lgr5 or Lgr6 rescue constructs and incubated with Wnt3A and Rspo-1 as indicated. Wnt3A readily induced TOPflash reporter activity without showing effects of removal of Lgr4. Rspondin potentiated the Wnt3A response, but this effect was sensitive to removal of Lgr4. Partial to virtually complete rescue was obtained with Lgr4, Lgr5, and Lgr6 rescue constructs.

Figure 6: Rspo-1-Lgr5 interaction K_D determination by Surface Plasmon Resonance array imaging. Anti-FLAG antibody spotted on the sensor chip, readily captured Rspo-1-FH. Results are given as overlay plot of three injection series of first huRSPO1-FH followed by huLGR5-Fc. Undiluted huRSPO1-FH at 1 µg/ml is injected after 300 s baseline. Saturation of the anti-Flag spot is within 2 minutes and a wash with running buffer resulted in a stable second baseline that shifted 28 millidegree which corresponds to a huRSPO1-FH ligand density of ~280 pg/mm^2. Three injections of huLGR5-Fc of 45 nM, 23 nM and 11 nM at 900 s resulted in a specific binding of LGR5-Fc on top of the RSPO1-FH and the exponentials were fitted with a discrete 1:1 interaction model. The residual plot shows a +2 till -2 mdegree experimental minus fit curve (perfect fit will give instrumental noise only) and the apparent KD of 3 nM is an average value calculated from a distribution of affinities and deviation to the 1:1 interaction model.

Fig 7 Direct physical interaction of Rspondin1 with Lgr4/5/6 exodomains

Fig 7A: Rspondin1 binds to Lgr4, Lgr5 and Lgr6. Top panel: HEK293T cells transfected ("tx") with tagged versions of the indicated proteins, incubated with Rspo1-Fc fusion protein at ~1 µg/ml. Cells were washed, lysed and Rspo1-Fc was immunoprecipitated with proteinG beads. Top: Western blotting for FLAG revealed specific binding to Lgr4, Lgr5 and Lgr6. Bottom: Input of tagged proteins.

Fig 8. Lgr4 is essential for transmitting Rspondin1 signals but dispensable for transmitting Wnt3A signals.

Fig 8A: Expression and downregulation of Lgr4 in HEK293T cells. Left: Northern blot for Lgr4 and Lgr5 on Ls174T and HEK293 cells as indicated. Right: Downregulation of

Lgr4 by three siRNAs (A, B, C) targeting the 3' UTR of Lgr4, or a control siRNA (I). Expression assessed three days post-transfection (top). Ethidiumbromide-stained gel as loading control (bottom).

Fig 8C: TOPflash Wnt reporter assays. As in Fig 5B. Rescue performed with wt Lgr5 (ERG) or mutant Lgr5 (ENG) constructs. Rescue was obtained both with wt and mutant Lgr5 constructs.

Fig 9. Rescue of Lgr4/5 deletion in cultured crypt organoids by Wnt signals.

Fig 9A: Organoids established under standard conditions[15] from *Lgr4*$^{lk/lk}$ *Lgr5*$^{fl/fl}$ *Villin-CreERT2* mice[36] or from control *Villin-CreERT2* mice. Tamoxifen treatment leads to death (asterisks) of mutant organoids but not of controls. This is overcome by addition of CHIR99021 at 5 μM.

Fig 9B: When CHIR99021 is subsequently withdrawn from *Lgr4/5* deleted organoids after passage, they immediately undergo apoptosis.

Fig 9C: Organoids established from *Lgr4*$^{lk/lk}$ *Lgr5*$^{fl/fl}$ *Villin-CreERT2* mice or from control *Villin-CreERT2* mice are infected with Wnt3-expressing retrovirus which turns organoids into growing, rounded cysts[34], or with control retrovirus. Subsequent deletion of *Lgr4* and *-5* has no effect on Wnt3-expressing organoids but leads to death of control organoids.

Fig. 10. Early and late lineage tracing in *Lgr4*$^{GFP\text{-}ires\text{-}CreERT2}$ *x R26R-LacZ* Cre reporter mice. Generation of the *Lgr4* allele was identical to that of the *Lgr5* and *Lgr6* alleles. Lineage tracing was performed and analyzed at 2 days post tamoxifen induction for scoring transit amplifying cell tracing and at 395 days post-induction for scoring stem cells. While most early tracing events resulted in marking single cells in crypts and villi (top panel), which disappeared over the next few days, significant numbers of stem cell tracing events were also noted as "blue ribbons" (middle panel: whole mount; lower panel: paraffin section histology. LacZ tracing in blue, tissue counter-stained in red). No tracing events were observed without tamoxifen induction.

Fig. 11 Design of *Lgr5*^{fl} allele.

Fig 11A: Black triangles indicate the inserted *loxp* sites. *PMCNeo* was removed by cre-mediated deletion in the germline.

Fig. 11B: PAS stain 5 days after gene deletion shows no effects on differentiated compartments. PAS staining of small intestine of mice of the indicated genotype, 5 days after gene deletion. Dark counterstain. Goblet cells: dyed body on villi; enterocyte: dyed apical membrane; Paneth cells: dyed cell body at crypt bottoms. Top panel is indistinguishable from wt intestine. Bottom panel: Arrows point to Paneth cell "nests" that remain after disappearance of proliferative crypt cells.

Fig. 12 Stem cells are still present one day after deletion of *Lgr4* and *Lgr5*.

Top/Middle: Ki57 stain for proliferative cells; light counter-stain. One day after deletion of *Lgr4* and *Lgr5*, mutant intestine (middle) is undistinguishable from wt intestine (top). Arrows indicate proliferative stem cells.

Bottom: Representative EM images of crypt base of an adult mouse homozygous for *Lgr4*^{fl} and *Lgr5*^{fl}, and carrying the *Ah-Cre* transgene analyzed one day (left) and five days (right) post-ß-naphtoflavone-induced deletion. Stem cells (S) are physically present at one day between the Paneth cells, but absent at day 5. Note extensive RER and secretory granules in Paneth cells (P)

Fig. 13 All Rspondins bind to Lgr4, Lgr5 and Lgr6. Rspondin1-4 were produced as IgFc fusion proteins and subjected to the assay depicted in Fig 9A. As is evident, all four Rspondins bound specifically to FH-tagged versions of human Lgr4, -5 and -6 transfected into HEK293T cells, but not to control Lgr7. Top: input of the four Lgr5 proteins. Bottom: Pull-down with Rspo1-Fc protein. Only the full-length Lgr5 protein binds Rspo1.

Fig. 14 N-terminal domains of Lgr5 are required for Rspo1 binding.

Fig 14A: Same assay strategy as in Fig 15. FL: full-length. ΔN: Deletion of the extreme N-terminus of Lgr5 (amino acids: 28 -50). ΔLRR1: deletion of the first LRR domain

from Lgr5 (amino acids: 51-75), ΔLRR1-17: Deletion of N-terminus and entire LRR region. Only full-length protein binds to Rspo1.

Fig 14B: Same as in A) but incubations with Rspo1 were performed after cell lysis.

Fig 15: Rspondin1-Lgr5 interaction KD determination by Surface Plasmon Resonance array imaging. Plot of the apparent kd and KD calculated values using a 1:1 binding model from serial injections of Lgr5-Fc to the array of RSPO-1 captured spots as measured in the IBIS MX96 instrument of IBIS Technologies (Enschede, The Netherlands). In IBIS-SPRint software (version 2) an exponential fitting routine y = a*ebx was used for extrapolating the values of KD and kd at Rmax=0 (arrows). The coëfficient of the exponential a (y-intercept) resulted in kd = 1.55 * 10-4 s-1, KD = 3.07 * 10-9 M respectively (ka = 5.04 * 104 M-1s-1) for binding of the Lgr5-Fc to RSpo-1-FH.

Method: IBIS-SPRinT 2.0. An array of anti-Flag spots with decreasing densities was printed on a preactivated sensor surface (IBIS Technologies, Enschede, the Netherlands) using a continuous flow microspotter (CFM) (Wasatch Microfluidics, Utah, US). Undiluted RSPO-1 -FH supernatant was exposed to the array and each anti-Flag spot captured a decreasing density of RSPO-1-FH as shown in the sensorgram of figure X. After measuring a new baseline for each spot the LGR5-Fc was injected and the sensorgram of each spot was recorded simultaneously. Rmax values were correlated to ligand (RSPO-1) densities.

The apparent rate- and affinity constant kd, ka and KD may change significantly, if rebinding effects, biphasic behavior and/or steric hindrance of the immobilized/captured molecules occurs at the sensor chip surface. A common strategy is to load the ligand at a very low density to mimic homogeneous binding in solution, preferably just above the limit of detection of the instrument. However, also at low ligand densities, the negative influence of rebinding etc. to the rate- and affinity constants kd, ka and KD may occur.

Here we apply a method for the determination of kd, ka and KD, which uses all biomolecular interaction data of LGR5 to RSPO-1 responses of the different spots, resulting in a single rate- and affinity constant kd , ka and KD at Rmax = 0 by exponential fitting the apparent kd and KD values to Rmax = 0. Theoretically, this

means that the affinity constant of a single ligand molecule to its single analyte molecule is determined in the limit to zero response.

Fig. 16 Dose response curve of Rspondin in Wnt reporter assay.

Experiment performed in HEK293T cells as in Figure 10, with constant amount of Wnt and increasing concentrations of Rspondin1. Transfections were done in duplicate; both values are given.

Fig. 17 Only Lgr4, 5, or 6 can rescue Rspondin responsiveness.

A) Assay performed as in Fig 10B. No rescue was obtained with *Lgr1*, *Lgr7*, or *Lgr8*

Fig. 18. Rescue of Lgr4/5 deletion in cultured crypt organoids by retrovirally expressed Lgr5 .

Representative images of organoids established from *Lgr4*$^{fl/fl}$ *Lgr5*$^{fl/fl}$ *Villin-CreERT2* mice are infected with retrovirus expressing GFP (top), Lgr5 (not shown) and Lgr5-ENG (bottom). Both Lgr5-expressing retroviruses protected against subsequent deletion of *Lgr4* and *-5*, implying that G protein signaling is not essential for Lgr signaling. Arrows indicate growth of rescued organoids.

Fig. 19 Lgr5 binds to the Furin like domain of RSp4

19A: shows the immunoprecipitation of flag tagged human RSpo4 proteins. hRSPO4 WT (Full length); hRSP4 daa (deletion of the basic amino acid domain); hRSPO4 dtht+ aa (deletion of the thrombospondin domain and Basic domain. These proteins are used to precipitate either hLGR5 (Fc tagged) or DKK (flag tagged). It was found that Lgr5 but not Dkk binds to hRSPO4 and its deletion that include only the furin domain. This indicates that Lgr5 binds to the furin domain.

19B: A flag tag Western blot shows equal expression of all RSPO proteins.

19C: A diagram showing the lanes used in the Western blot.

Figure 20: Activation of B-Cat/TCF TOP promoter by Wnt, RSpo and LGR5 antibodies.

Fig 20A: HEK293T cells stably transfected with LGR5 are transfected with the TOP reporter and Renilla reporter constructs. One day later the cells are stimulated with: 1) Wnt3A condition medium (Produced in L-Cells and added at 1/3 of the total medium volume), 2) RSpo His tagged purified on a nickel column from 293T cells added at 1ug/ml, 3) Anti LGR5 Rat monoclonal antibodies 1D9, 4D11, 8F2 prot A purified at 1ug/ml. Luciferase activity is determined 24h after stimulation. The luciferase counts represent the level of activation of the Wnt pathway compared to the control.

Fig 20B: HEK293T cells were seeded into 96-well plates in DMEM/10% FCS at a density of 10^4cells/well and in triplicate transfected (PEI: Polyethylenimine, linear, MW-25,000) with 10 ng TOP or FOP luciferase (Ref 1), 1 ng TK Renilla, 10 ng of pcDNA-based hLgr5-Flag, and 80 ng empty vector DNA. After 24 hrs, medium was replaced for 50% fresh DMEM/10% FCS, and 50% Wnt3a-conditioned medium or control conditioned medium. At the same time point, purified human Rspondin1 (stock solution of 100 µg/ml in PBS) was added at a final concentration of 1 µg/ml and ProtA-purified (stock solution of 1mg/ml in PBS) Lgr5-specific antibodies (1D9 and 8F2) at three different concentrations. At 72 hrs results were measured using a dual luciferase assay kit (Promega USA). The luciferase counts represent the level of activation of the Wnt pathway compared to the control.

Fig. 21 Graph showing effect of anti-Rspo3 mAb on Wnt activity

Blocking of RSpondin-induced enhancement of Wnt signaling by rat anti h/m Rspondin3 antibody;

HEK293T cells were transfected with Wnt pathway-specific (TOP) luciferase reporter in combination with a TK driven Renilla reporter. After 24 hrs cells were incubated for 24 hrs. with control conditioned medium (CM), Wnt3a CM, or combinations of Wnt3a + Rspondin-CM. The relative volume proportion (%) of Rspondin CM is indicated. A rat monoclonal antibody (R&D systems, clone 400403), recognizing both human and mouse Rspondin3, was added to a final concentration of 2.5 µg/ml. Wnt pathway activity was

quantitated by comparing averages of triplicate Renilla-normalized TOP luciferase counts of cells receiving stimuli, to un-stimulated cells.

Fig. 22 Isolated furin domains enhance Wnt signaling to a greater extent than full-length Rspondin

The graph shows TOP luciferase counts relative to control (y-axis) for full length Rspo1, compared to truncated fragments of Rspo1, as described below. Fragment FUR1/2+Thr is represented by SEQ ID NO: 139 and fragment FUR1/2 is represented by SEQ ID NO: 140. The numbers represent the lanes on the x-axis.

1 = ctr medium	
2= Wnt3a	
3= Wnt3a + Rspo1Fc-conditioned medium	
5= Wnt3a FL Rspo1-HIS	62 nM
6=	30 nM
7=	15 nM
8=	7 nM
9=	3 nM
10=	1.5 nM
5= Wnt3a FUR1/2+Thr-HIS Peng	62 nM
6=	30 nM
7=	15 nM
8=	7 nM
9=	3 nM
10=	1.5 nM
5= Wnt3a FUR1/2-HIS	62 nM
6=	30 nM

7=	15 nM
8=	7 nM
9=	3 nM
10=	1.5 nM

Fig. 23 Antibody 1D9 can be used as an Rspondin mimic in culture media

This figure shows mouse colon organoids grown in Sato medium (Sato et al. Nature 459, 262-5, 2009). Left column show organoids grown in the presence of full length RSP1. Middle column shows the media with supplemented with the 1D9 antibody instead of RSP1. Right column shows organoids grown without RSP1 or 1D9.

Fig. 24 Epcam-Ab-Rspondin1 furin domain fusion (a bispecific agonist targeting epithelial cells) is effective in culture media at lower concentrations than Rspondin1 alone.

This figure shows mouse colon organoids grown in Sato medium (Sato et al Nature 459, 262-5, 2009). Left column show organoids grown in the presence of full length RSP1. Middle column has the EpcamAb-RSP furin domain fusion at a concentration 50x lower than the full length RSP1 in the left column. The right column shows organoids without RSP1 or the fusion protein.

Fig. 25 Epcam-Ab-Rspondin1 furin domain fusion in a TOP/FOP assay

This graph shows the results of a TOP/FOP assay for the multi-targeting compound, EpcamAb-RSP furin domain fusion, compared to normal wild-type Rspondin1. The luciferase counts on the y-axis are a measure of Wnt pathway activation compared to a control. This figure shows that Epcam-Ab-Rspondin1 furin domain fusion mimics the activity of Rspondin for enhancing Wnt activation at a 16x lower concentration than Rspondin.

Fig. 26 1D9 antibody binds the CRL region of LGR5

This figure demonstrates that the 1D9 antibody binds the CRL region of LGR5. Purified proteins and antibodies were used. LGR5-543 represents the full exo domain of LGR5

(CRL + Leucine rich repeats). LGR5-478 represents the exo domain of LGR5 without the CRL region.

This graph represents the protein complexes separated by size on a gel filtration column. On the left are the proteins running of the column first (so the biggest complexes). From left to right we have a blue line with two peaks. The left peak represents the LGR5-543/RSp1/Antibody 1D9 complex. The next peak of the same line is the LGR-543/RSp complex (added in excess). The next separation represented by the second line from the left having only a single peak is the antibody alone (black line). Below this the separation of the LGR5-478 incubated with the Antibody. This purification shows that the antibody purifies with the Ab alone peak. The next peak on the same line is at the size of the Lgr5-RSp peak. This demonstrates that the Ab did not bind the complex (green line). Last the middle sized peak (amplitude) on the right represent the Lgr5/RSp complex. This line has a single peak (Red line).

Fig. 27 Lgr is expressed on the basolateral side of the membrane

The figures show fluorescent staining of his-tagged recombinant fusion Lgr5 (left) and Lgr4 (middle) compared to the control (right), in mouse colon organoids grown in Sato medium (Sato et al. Nature 459, 262-5, 2009). It can be seen that Lgr proteins are expressed on the basolateral (non-lumen) side of the membrane.

Fig.28 TOP/FOP assay showing Lgr4 and Lgr5 antagonists

This experiment is done with the stable transfected HEK-Lgr5FlagHA or HEK-Lgr4FlagHA cells. All cell have been transfected with the Wnt activity reporter TOP/FOP. Wnt activity was tested with and without several Lgr5 and Lgr4 rat monoclonal antibodies.

28A: Lgr5 antibodies - From left to right: nothing added (control); Wnt only added; Wnt + RSp1; Wnt+ RSpo + Antibody medium without antibody; Wnt + all antagonistic antibodies; far right-hand column shows Wnt + the agonistic antibody 1D9.

28B: Lgr4 antibodies - From left to right: nothing added (control); Wnt only added; Wnt+ RSpo + Antibody medium without antibody; Wnt + all antagonistic antibodies;

MODES FOR CARRYING OUT THE INVENTION

Various aspects and embodiments of the present invention will now be described in some detail. It will be appreciated that modification of detail may be made without departing from the invention.

Example 1: Lgr4/5/6 family members reside in Frizzled-Lrp complexes and mediate signaling by the Wnt agonist Rspondin1

The role of Lgr proteins in stem cells was previously unknown. Similarly, whilst a number of contrasting explanations had been postulated, the exact mechanism by which Rspondin activates the Wnt/β-signalling was also unknown. In this first example, the inventors describe the surprising discovery that Lgr proteins, which are Wnt target genes, also play a role themselves in the Wnt signalling pathway and form part of the Wnt receptor complex. Furthermore, the inventors demonstrated that Rpsondin binds to Lgr proteins in the Wnt receptor complex. The inventors thus describe an unexpected new mechanism for Rspondin-mediated Wnt/β-catenin signalling activation. This provides new avenues for identifying agonists for the Wnt/β-catenin signalling pathway, which mimic the Rpsondin-Lgr interaction.

The adult stem cell marker Lgr5 and its relative Lgr4 are often co-expressed in Wnt-driven proliferative compartments. In summary, the inventors find that conditional deletion of the two genes in the gut impairs Wnt target gene expression and results in rapid demise of intestinal crypts, thus phenocopying Wnt pathway inhibition. Mass-spectrometry demonstrates that Lgr4 and Lgr5 associate with the Frizzled/Lrp Wnt receptor complex. Each of the four Rspondins, secreted Wnt pathway agonists, can bind to Lgr4, -5 and -6. In HEK293 cells, Rspondin1 enhances canonical Wnt signals initiated by Wnt3A. Removal of Lgr4 does not affect Wnt3A signaling, but abrogates the Rspondin1-mediated signal enhancement, a phenomenon rescued by re-expression of Lgr4, -5 or -6. Genetic deletion of Lgr4/5 in intestinal crypt cultures phenocopies withdrawal of Rspondin1 and can be rescued by Wnt pathway activation. Lgr5 homologs are facultative Wnt receptor components that mediate Wnt signal enhancement by soluble Rspondin proteins.

Lgr4, *Lgr5* and *Lgr6* encode orphan 7-Transmembrane receptors, that are close relatives of the receptors for the hormones FSH, LH and TSH[101]. It was previously unknown how the Lgr4-6 receptors signal. *Lgr5* is a Wnt target gene which marks proliferative stem cells in several Wnt-dependent stem cell compartments, specifically the small intestine and colon[102], the stomach[103], and the hair follicle[104]. *Lgr6* marks multipotent stem cells in the epidermis[105]. The expression of *Lgr4* is much broader[106], but the inventors noted that *Lgr5* is co-expressed with *Lgr4* in the stem cell compartments mentioned above. For instance, *Lgr5* marks small intestinal stem cells at the base of crypts, while *Lgr4* marks all crypt cells, including the *Lgr5*$^+$ stem cells (Fig 1A). Both, *Lgr4* and *Lgr5* null mutations are neonatal-lethal in mice[107, 108].

Lgr5 and Lgr4 are often co-expressed in Wnt-driven proliferative compartments. To address a potential function in crypts, the inventors generated the *Lgr5*^{fl} allele (Fig 1B) in which exon 16 is flanked by *loxp* sites; its deletion causes a frame shift. This allele was crossed into a mouse strain carrying conditional *Lgr4*^{fl} alleles[108] and the gut-specific *Ah-Cre* transgene, which is inducible by β-naphtoflavone[109]. Conditional deletion of *Lgr5* alone in the intestinal epithelium of adult mice yielded no apparent phenotype. In particular, the Paneth cell phenotype reported previously in *Lgr5* null neonatal mice[110] could not be confirmed. Deletion of *Lgr4* alone resulted in a deleterious effect on the proliferative cells in crypts, which typically became obvious from day 4-5 post-induction onwards. Crypt proliferation halted and many crypts disappeared. No direct effects were observed on the differentiated compartments of the villus. The combined deletion of *Lgr4* and *-5* enhanced the crypt phenotype as judged by the cell proliferation marker Ki67 and the stem cell marker Olfm4[111]. Fig 1C depicts typical results obtained at day 5 post-induction. Over the next few days, villi typically shortened because of the halted cell production in crypts and eventually the phenotype was not compatible with life.

Two signalling pathways, Wnt[112, 113] and Notch[114], are crucial for the maintenance of adult crypt proliferation. To determine whether Lgr4/5 genes may be involved in these signalling pathways, the inventors performed differential gene expression analysis by microarray on small intestinal crypts isolated from Lgr4/5 knock-out mice. To address immediate changes in gene expression, the gene expression analysis performed on day 1

post-deletion of *Lgr4* and *-5*, before any histological changes are apparent. Simultaneous deletion of Lgr4 and Lgr5 resulted in the significant downregulation of 307 genes in two separate experiments (gene identities are shown in Table 2).

Table 2 shows 306 unique genes significantly downregulated in Lgr4/5 double knock-out mice one day after deletion in two biological replicates performed as a dye swap experiment, resulting in 4 individual arrays:

		Biological Replicate 1		Biological Replicate 2			
	Probe Name	Dye swap 1	Dye swap 2	Dye swap 1	Dye swap 2	Avg	GeneName
1	A_52_P87997	2.13	1.39	3.90	3.90	2.83	Gsdmc2
2	A_51_P150489	1.76	2.21	3.44	3.69	2.77	Gsdmc1
3	A_51_P521155	2.25	2.11	3.42	3.25	2.76	1700124P09Rik
4	A_52_P498798	2.36	2.10	2.99	3.09	2.64	Gsdmc3
5	A_51_P312336	2.57	2.97	2.45	2.44	2.61	Slc14a1
6	A_52_P25357	3.06	3.06	1.98	1.82	2.48	NAP027049-1
7	A_52_P387458	1.65	1.55	3.29	3.14	2.41	1810035L17Rik
8	A_51_P337308	0.85	2.61	2.78	3.30	2.39	Saa3
9	A_51_P179878	3.49	2.69	1.90	1.42	2.38	2900019G14Rik
10	A_51_P501840	2.10	2.75	2.10	1.94	2.22	Dnajb3
11	A_52_P251690	1.64	2.40	2.34	2.48	2.22	Gvin1
12	A_51_P267700	2.52	2.65	1.89	1.81	2.22	1190003M12Rik
13	A_52_P566963	0.93	1.67	2.90	3.12	2.15	Msi1
14	A_51_P515605	1.31	2.39	2.51	2.34	2.14	Col3a1
15	A_52_P67463	1.51	0.62	3.51	2.87	2.13	1810026B05Rik
16	A_52_P1016836	1.56	1.96	2.50	2.45	2.12	ENSMUST00000061000
17	A_52_P403157	1.86	2.83	1.90	1.75	2.09	Sorbs2
18	A_52_P503387	0.69	1.32	3.15	3.16	2.08	Trp53inp1
19	A_51_P225493	1.66	1.09	2.91	2.55	2.05	1700016C15Rik
20	A_51_P117952	1.15	1.32	3.01	2.71	2.05	Hspa1a
21	A_51_P285097	0.98	2.15	2.20	2.84	2.04	Wdr38
22	A_51_P218091	2.24	2.80	1.53	1.48	2.01	Lgr5
23	A_52_P488919	1.34	1.72	2.60	2.32	1.99	Zbtb26
24	A_51_P264685	0.59	1.38	3.02	2.97	1.99	Whrn
25	A_51_P347547	1.30	1.51	2.50	2.64	1.99	Klf10
26	A_51_P450752	1.41	3.61	1.34	1.57	1.98	Pla2g4b
27	A_52_P522166	0.63	1.06	3.03	3.17	1.97	TC1654900
28	A_52_P134195	1.39	3.83	1.30	1.28	1.95	Ceacam10
29	A_52_P668812	0.85	1.23	2.66	3.03	1.94	Sgol2
30	A_51_P182303	1.36	2.26	2.06	2.06	1.93	Col1a2

31	A_51_P130544	1.34	0.95	2.79	2.47	1.89	Gas5
32	A_52_P828218	1.78	2.36	1.44	1.94	1.88	Olfm4
33	A_52_P195772	1.70	1.68	1.96	2.19	1.88	Rbmx
34	A_52_P682465	1.84	1.17	2.39	2.07	1.87	Rps28
35	A_52_P190744	0.94	1.96	2.18	2.37	1.87	Rnpc3
36	A_51_P507602	1.02	2.24	2.06	2.15	1.86	Clca4
37	A_52_P386075	1.19	1.19	2.36	2.67	1.85	ENSMUSG00000057445
38	A_51_P480709	1.39	1.61	2.10	2.28	1.84	Ywhaz
39	A_52_P460584	1.14	2.45	1.91	1.81	1.83	Tnfrsf25
40	A_52_P367621	1.25	1.28	1.82	2.80	1.79	Arid5b
41	A_51_P478486	0.80	0.73	2.32	3.18	1.76	Fus
42	A_51_P185660	1.99	1.95	1.82	1.26	1.75	Ccl9
43	A_52_P222230	1.50	1.54	1.80	2.16	1.75	AK157581
44	A_52_P1052476	1.25	1.71	2.01	2.02	1.75	Bub3
45	A_51_P447189	1.21	1.15	2.29	2.34	1.75	1700030C10Rik
46	A_52_P145433	1.64	0.93	2.53	1.86	1.74	D7Ertd715e
47	A_51_P324161	0.67	1.17	2.18	2.84	1.72	Ankrd26
48	A_52_P780821	1.25	0.77	2.20	2.60	1.71	Msi2
49	A_51_P285413	1.00	1.98	1.58	2.20	1.69	Rbbp6
50	A_52_P540219	1.06	2.29	1.48	1.91	1.69	Timp2
51	A_52_P211418	0.80	0.91	2.11	2.90	1.68	6030408C04Rik
52	A_52_P328825	2.24	2.55	0.92	0.90	1.65	Wdfy1
53	A_52_P529790	1.88	2.07	1.24	1.43	1.65	Hisppd2a
54	A_52_P100002	1.32	0.92	2.41	1.97	1.65	Rps24
55	A_51_P460391	1.38	1.38	2.04	1.75	1.64	Defcr21
56	A_52_P355004	1.87	2.80	1.16	0.72	1.64	Cttnbp2
57	A_52_P419879	0.68	1.81	1.55	2.50	1.63	TC1695874
58	A_52_P309022	1.53	1.17	1.89	1.94	1.63	Dach1
59	A_51_P162984	1.83	0.99	1.92	1.78	1.63	Prm1
60	A_51_P129464	1.04	1.37	2.01	2.09	1.63	Scd2
61	A_52_P522157	0.76	0.68	2.63	2.41	1.62	2810026P18Rik
62	A_52_P214630	0.92	1.41	2.24	1.91	1.62	Sox9
63	A_52_P276727	1.74	1.54	1.72	1.46	1.62	Lgr4
64	A_52_P14778	1.23	1.81	1.67	1.73	1.61	Piwil4
65	A_51_P275527	1.12	1.89	1.63	1.79	1.61	Slc12a2
66	A_52_P1133481	1.05	1.96	1.85	1.56	1.61	BF149456
67	A_51_P508510	0.93	1.94	1.56	1.97	1.60	Notch1
68	A_52_P483885	1.22	1.58	1.92	1.67	1.60	4930455C21Rik
69	A_52_P679105	1.96	1.66	1.60	1.14	1.59	Prss23
70	A_51_P101719	0.70	0.82	2.28	2.56	1.59	Ttc14
71	A_52_P431615	1.32	1.98	1.23	1.81	1.58	Gm1966
72	A_51_P281326	1.28	1.87	1.54	1.57	1.57	Clasp2
73	A_52_P659448	1.88	1.11	1.44	1.83	1.56	91304300000
74	A_52_P510119	0.89	0.79	2.09	2.45	1.55	Pgm2l1

75	A_51_P375543	1.28	1.18	1.67	2.08	1.55	Myb
76	A_52_P407007	1.22	1.15	1.57	2.26	1.55	4632415L05Rik
77	A_52_P381009	0.78	0.99	1.99	2.45	1.55	Tia1
78	A_52_P306845	0.75	1.50	2.11	1.83	1.55	Cav1
79	A_51_P114287	1.02	0.80	2.18	2.16	1.54	Cpsf6
80	A_51_P346715	0.88	1.30	1.89	2.09	1.54	D4Wsu53e
81	A_51_P463765	1.11	2.19	1.43	1.42	1.54	Timp3
82	A_52_P16136	1.07	0.75	2.10	2.21	1.53	D10Bwg1070e
83	A_52_P35534	2.24	2.51	0.78	0.59	1.53	E230029C05Rik
84	A_51_P222936	0.86	2.38	1.45	1.42	1.53	Fmnl2
85	A_52_P119039	0.93	1.24	1.75	2.14	1.52	Hmgcs1
86	A_51_P398525	1.37	2.70	0.97	1.02	1.51	Fn3k
87	A_53_P71261	0.82	0.74	2.27	2.21	1.51	TC1615264
88	A_51_P172688	1.25	1.26	1.78	1.75	1.51	Stard3nl
89	A_51_P201254	0.59	0.94	2.16	2.30	1.50	AK047739
90	A_52_P465809	0.92	0.94	1.91	2.18	1.49	Pwwp2a
91	A_51_P501757	0.82	1.64	1.68	1.78	1.48	Rgmb
92	A_52_P566487	0.61	1.14	2.19	1.96	1.48	Zfp26
93	A_51_P221031	1.29	1.93	1.14	1.46	1.46	Slc16a12
94	A_52_P531514	1.64	1.44	1.29	1.42	1.45	Uhrf2
95	A_52_P81571	0.81	2.11	1.10	1.75	1.44	Rad51l1
96	A_52_P367294	0.94	0.82	1.85	2.17	1.44	Fsd1l
97	A_52_P566840	1.31	2.52	0.81	1.14	1.44	Gpr110
98	A_51_P265806	2.13	1.26	1.37	1.00	1.44	Clca2
99	A_51_P449624	0.71	1.06	1.65	2.33	1.44	6430706D22Rik
100	A_51_P268167	0.75	1.44	1.64	1.91	1.44	D14Abb1e
101	A_51_P390755	1.34	2.35	0.75	1.22	1.42	9630010G10Rik
102	A_52_P229536	1.12	0.90	1.67	1.90	1.40	Cd44
103	A_52_P172704	1.17	1.58	1.31	1.52	1.39	E030011O05Rik
104	A_52_P18116	1.19	0.99	1.69	1.69	1.39	Ccl24
105	A_51_P433796	1.81	1.35	1.29	1.07	1.38	Agr3
106	A_52_P175190	0.99	2.86	0.79	0.84	1.37	Egfr
107	A_51_P211165	0.77	0.63	1.97	2.12	1.37	Rbm26
108	A_51_P374707	1.22	1.50	1.42	1.34	1.37	Tspan12
109	A_52_P288177	1.29	1.28	1.27	1.62	1.36	Epha4
110	A_52_P655285	1.37	1.10	1.46	1.52	1.36	Zfp462
111	A_51_P483483	1.12	1.18	1.68	1.43	1.35	ENSMUST00000038450
112	A_52_P247927	0.98	1.78	1.42	1.22	1.35	2810442I21Rik
113	A_51_P474902	0.71	2.01	1.18	1.49	1.35	Acvr1c
114	A_51_P426919	0.87	0.89	1.89	1.71	1.34	Gt(ROSA)26Sor
115	A_52_P507479	1.84	1.39	0.82	1.25	1.33	Fam73a
116	A_51_P335000	1.82	0.92	1.49	0.99	1.30	Fhl1
117	A_52_P308875	1.41	1.90	0.93	0.98	1.30	Apobec3
118	A_51_P430071	0.95	1.14	1.61	1.52	1.30	Hist2h2be

119	A_52_P290325	0.71	1.25	1.58	1.67	1.30	Depdc1a
120	A_51_P170725	1.08	1.59	1.30	1.22	1.30	1300002K09Rik
121	A_52_P187987	1.52	1.35	1.14	1.16	1.29	2310001H17Rik
122	A_52_P486260	1.97	1.57	0.96	0.66	1.29	Prelp
123	A_52_P18706	1.53	1.22	1.01	1.33	1.27	Dppa5a
124	A_51_P120066	1.54	0.70	1.52	1.32	1.27	9330151L19Rik
125	A_51_P517843	1.36	1.41	1.12	1.13	1.26	Glipr2
126	A_52_P60194	1.49	1.15	1.09	1.31	1.26	C4bp
127	A_52_P639343	0.94	1.69	1.11	1.28	1.26	D330028D13Rik
128	A_51_P474538	1.00	0.75	1.43	1.84	1.25	Igf1r
129	A_52_P669035	0.95	1.51	1.28	1.27	1.25	Clca1
130	A_52_P79648	0.66	0.93	1.72	1.70	1.25	Ocm
131	A_52_P25932	0.80	1.22	1.32	1.66	1.25	TC1660846
132	A_52_P363110	0.85	1.57	1.25	1.33	1.25	Fgfr2
133	A_52_P674165	0.95	0.93	1.89	1.23	1.25	Rsbn1l
134	A_51_P105178	1.13	0.88	1.40	1.59	1.25	Sox4
135	A_52_P652293	1.59	1.37	1.25	0.78	1.25	Trim24
136	A_52_P377791	1.22	1.24	1.40	1.10	1.24	Fxyd3
137	A_52_P652289	0.62	0.69	1.68	1.96	1.24	AK032921
138	A_51_P492707	1.91	1.24	0.93	0.87	1.24	Ptpro
139	A_52_P239536	1.00	0.97	1.47	1.50	1.24	Ppp1r9a
140	A_52_P425317	1.44	0.88	1.31	1.32	1.24	4933406C10Rik
141	A_52_P67570	0.73	1.18	1.27	1.75	1.23	Lass4
142	A_52_P454703	0.64	1.41	1.33	1.54	1.23	BC033430
143	A_51_P327369	0.82	0.95	1.43	1.70	1.23	Plekhg3
144	A_52_P641684	0.79	1.15	1.37	1.60	1.23	Nfia
145	A_52_P479539	0.81	0.93	1.71	1.41	1.22	Cit
146	A_51_P323248	0.99	0.80	1.57	1.50	1.22	Sdc4
147	A_52_P491849	0.65	0.60	1.64	1.95	1.21	Trp53
148	A_52_P742248	0.64	0.58	1.77	1.86	1.21	Als2cr13
149	A_51_P394172	1.21	1.87	0.83	0.93	1.21	Es1
150	A_51_P168439	1.16	1.19	1.34	1.13	1.20	Klhl24
151	A_52_P661071	1.03	0.63	1.57	1.57	1.20	Snhg3
152	A_51_P409694	0.90	1.87	0.96	1.06	1.20	Psrc1
153	A_52_P481770	1.55	0.66	0.90	1.67	1.19	NAP103155-1
154	A_52_P260747	0.97	1.60	1.20	1.00	1.19	E430016P22Rik
155	A_52_P130044	0.75	0.60	1.50	1.89	1.18	TC1632596
156	A_51_P244586	1.05	1.12	1.14	1.40	1.18	Zfp618
157	A_51_P110301	0.83	1.54	1.20	1.13	1.18	C3
158	A_51_P358445	0.87	1.15	1.34	1.34	1.17	Dnase1
159	A_51_P517672	0.72	1.12	1.41	1.44	1.17	Rnf152
160	A_52_P61893	0.91	0.58	1.47	1.72	1.17	Nono
161	A_51_P277588	1.03	1.12	1.31	1.21	1.17	Sfrs18
162	A_51_P237924	1.00	0.83	1.13	1.69	1.16	Slc6a7

163	A_52_P1115511	1.76	1.15	0.96	0.77	1.16	6030422H21Rik
164	A_52_P10683	1.22	0.87	1.24	1.28	1.15	Hist1h1d
165	A_52_P996032	0.85	0.70	1.33	1.71	1.15	A630031M23Rik
166	A_51_P293729	0.97	0.65	1.39	1.58	1.14	Ivns1abp
167	A_52_P485417	0.84	0.73	1.29	1.70	1.14	Hmgb2
168	A_52_P254095	0.78	0.78	1.71	1.29	1.14	Cd200
169	A_52_P481493	1.01	0.72	1.33	1.50	1.14	Fkbp5
170	A_52_P244193	0.86	1.07	1.36	1.24	1.14	Cd24a
171	A_51_P489779	1.22	0.87	1.39	1.06	1.13	Cdk6
172	A_51_P352968	1.18	1.39	0.86	1.11	1.13	Marcks
173	A_52_P590701	0.73	1.51	1.31	0.98	1.13	Dzip3
174	A_51_P419726	1.37	1.13	1.04	0.97	1.13	Ptprs
175	A_51_P151516	1.32	1.72	0.74	0.73	1.13	B230208H11Rik
176	A_52_P232433	1.07	0.60	1.37	1.47	1.13	Hnrnpa1
177	A_51_P335770	1.12	1.39	1.17	0.81	1.12	Afap1
178	A_52_P355751	1.19	1.19	1.11	0.99	1.12	544988
179	A_52_P1148292	0.82	1.47	1.10	1.08	1.12	AK168746
180	A_52_P679152	0.64	0.90	1.40	1.51	1.11	Clk1
181	A_51_P364657	1.36	1.39	0.75	0.90	1.10	Rnf32
182	A_51_P509746	1.98	0.73	0.93	0.76	1.10	Porcn
183	A_51_P127681	1.04	1.28	1.10	0.96	1.10	Clic4
184	A_51_P102607	1.13	0.99	0.93	1.33	1.10	2310007H11Rik
185	A_51_P444696	0.62	0.70	1.34	1.71	1.09	Cdca2
186	A_52_P409076	1.23	1.41	0.81	0.91	1.09	Sema5a
187	A_51_P425962	0.77	1.05	1.44	1.10	1.09	Khdrbs3
188	A_52_P146848	0.59	0.69	1.56	1.51	1.09	BF120285
189	A_51_P250217	1.30	1.30	0.84	0.90	1.09	A_51_P250217
190	A_52_P93284	1.68	0.75	1.09	0.82	1.08	Mycl1
191	A_52_P666698	0.77	1.40	0.72	1.43	1.08	TC1715877
192	A_51_P181751	1.22	0.71	1.39	1.00	1.08	4930488E11Rik
193	A_51_P419319	1.04	0.84	1.15	1.28	1.08	Aqp4
194	A_51_P216605	0.75	1.20	1.31	1.04	1.08	Hbp1
195	A_52_P206003	1.69	1.31	0.73	0.67	1.07	Defcr23
196	A_52_P319161	0.87	0.59	1.31	1.48	1.06	Stmn1
197	A_52_P15073	0.61	0.92	1.24	1.46	1.06	4833442J19Rik
198	A_51_P408749	1.37	1.01	1.20	0.66	1.06	4933439C10Rik
199	A_52_P646112	0.76	0.63	1.47	1.36	1.06	Rps13
200	A_51_P472481	1.46	1.32	0.78	0.67	1.06	Dync2li1
201	A_51_P147651	0.73	0.71	1.10	1.63	1.04	Ccdc15
202	A_51_P173735	0.62	1.32	0.94	1.28	1.04	Dock11
203	A_51_P147714	0.62	1.22	1.05	1.25	1.03	Sfrs14
204	A_52_P65295	0.89	0.73	0.99	1.52	1.03	AK084936
205	A_52_P223508	0.73	1.46	0.99	0.94	1.03	6720401G13Rik
206	A_52_P162957	1.04	0.98	1.06	1.03	1.03	Frat2

207	A_51_P429197	0.70	0.66	0.98	1.76	1.03	Csnk1e
208	A_52_P555537	1.25	0.95	1.02	0.87	1.02	2810008D09Rik
209	A_51_P443322	0.82	0.85	0.91	1.50	1.02	Eif3c
210	A_52_P622850	0.85	0.80	1.42	0.99	1.02	Hes5
211	A_52_P557265	1.18	1.05	0.80	1.01	1.01	Cyp39a1
212	A_51_P104125	0.72	0.60	1.41	1.31	1.01	Fam76b
213	A_51_P303332	0.83	0.97	1.19	1.03	1.00	Mitd1
214	A_51_P404077	1.38	1.13	0.71	0.78	1.00	Fzd2
215	A_51_P225781	1.30	0.97	0.83	0.90	1.00	Insc
216	A_52_P251366	1.10	0.97	0.95	0.95	0.99	Neil3
217	A_52_P598309	0.73	0.81	1.21	1.22	0.99	1500012F01Rik
218	A_52_P124083	0.72	1.13	1.09	1.01	0.99	A530082C11Rik
219	A_52_P478289	0.60	0.81	1.23	1.29	0.98	C430048L16Rik
220	A_52_P569218	1.06	0.82	1.08	0.96	0.98	Utrn
221	A_51_P482043	0.88	0.72	1.24	1.05	0.97	Epm2aip1
222	A_51_P407726	1.10	1.08	0.84	0.86	0.97	Zan
223	A_51_P336599	1.46	0.79	0.85	0.78	0.97	Kcne3
224	A_52_P317246	1.62	0.75	0.67	0.84	0.97	Esrrg
225	A_51_P306017	1.59	0.71	0.72	0.85	0.97	Dll1
226	A_51_P199098	0.92	0.76	1.05	1.14	0.97	Tmem107
227	A_51_P488333	0.63	0.67	1.09	1.47	0.97	A130022F02Rik
228	A_52_P562872	0.66	0.90	1.06	1.24	0.96	4930520O04Rik
229	A_51_P184796	0.75	1.17	0.85	1.08	0.96	Zfp101
230	A_51_P288277	1.12	0.76	1.14	0.83	0.96	AK003987
231	A_51_P251438	0.96	0.99	0.91	0.99	0.96	9130004C02Rik
232	A_51_P434332	1.15	1.23	0.74	0.71	0.96	Sycn
233	A_52_P561073	1.16	0.99	0.82	0.86	0.96	Tox
234	A_51_P117666	0.76	0.61	1.18	1.27	0.96	1810032O08Rik
235	A_52_P650259	1.16	0.92	0.88	0.85	0.95	Noxa1
236	A_51_P337662	0.80	0.74	1.23	1.02	0.95	Ddx26b
237	A_51_P402760	0.67	1.45	0.74	0.93	0.95	Pla2g4a
238	A_51_P169564	0.79	0.66	0.86	1.47	0.94	5730455P16Rik
239	A_52_P139936	1.15	0.59	1.36	0.67	0.94	Phf21b
240	A_51_P261428	1.13	0.95	0.70	0.98	0.94	Pdcd4
241	A_51_P399305	0.90	1.26	0.65	0.93	0.93	Tnfrsf19
242	A_52_P440729	1.02	1.27	0.60	0.83	0.93	Atf7ip
243	A_51_P416846	1.15	1.02	0.84	0.72	0.93	2610020H08Rik
244	A_51_P217682	0.87	1.12	0.99	0.74	0.93	Pcf11
245	A_51_P101621	0.58	0.63	1.07	1.43	0.93	Creb1
246	A_51_P348325	1.04	0.81	0.65	1.20	0.93	Wdr51b
247	A_51_P155997	0.65	0.71	1.31	1.03	0.92	Bcl7a
248	A_51_P300337	1.18	0.97	0.76	0.78	0.92	Csrp2
249	A_51_P146126	0.78	0.66	1.49	0.77	0.92	Cdkn1b
250	A_52_P678800	0.90	0.81	1.01	0.97	0.92	Rps21

251	A_52_P329054	0.80	0.69	1.24	0.96	0.92	Cep70
252	A_52_P234039	0.85	0.84	1.18	0.80	0.92	Znrf3
253	A_52_P558368	0.98	0.90	1.05	0.74	0.92	Zdhhc1
254	A_52_P337246	0.81	1.39	0.71	0.75	0.92	Isl1
255	A_52_P301035	1.20	1.11	0.73	0.62	0.91	Rccd1
256	A_52_P90257	0.72	0.81	1.02	1.10	0.91	TC1668402
257	A_52_P148212	0.73	1.26	0.88	0.77	0.91	C79407
258	A_51_P366931	0.67	0.77	1.11	1.10	0.91	Prc1
259	A_52_P3825	0.96	1.07	0.73	0.90	0.91	Ddb2
260	A_52_P206762	0.73	0.86	1.04	1.01	0.91	Zfp422
261	A_51_P272283	0.59	1.02	0.98	1.06	0.91	Cmbl
262	A_51_P245796	1.18	1.10	0.61	0.74	0.91	Ddit4
263	A_51_P127315	0.95	0.84	1.04	0.80	0.91	Sesn3
264	A_52_P748067	1.28	0.78	0.85	0.71	0.91	Ai608034
265	A_51_P306608	0.93	0.62	1.08	0.96	0.90	Taf1d
266	A_51_P262230	0.63	0.72	1.39	0.82	0.89	a2ld1
267	A_52_P84037	0.59	0.76	0.93	1.23	0.88	Socs2
268	A_51_P157462	0.68	0.68	0.90	1.25	0.88	Rgn
269	A_51_P345248	0.68	0.63	0.59	1.59	0.87	Sfxn2
270	A_52_P416756	0.96	0.70	0.74	1.09	0.87	D17H6S56E-5
271	A_51_P142896	0.61	0.94	0.70	1.24	0.87	Cd59a
272	A_52_P495104	0.64	0.76	1.05	1.03	0.87	Heca
273	A_51_P439970	0.91	0.62	0.89	1.01	0.86	Ggh
274	A_52_P362997	0.90	1.12	0.58	0.82	0.86	Cenpl
275	A_52_P395929	0.59	0.91	0.97	0.95	0.86	TC1677080
276	A_51_P475785	1.06	0.63	0.98	0.75	0.85	BC052040
277	A_52_P613498	0.77	0.79	0.93	0.92	0.85	4833420G17Rik
278	A_51_P312482	0.83	0.78	1.09	0.69	0.85	Ccdc34
279	A_51_P355906	1.08	0.79	0.88	0.64	0.85	Kit
280	A_52_P556804	0.59	0.79	1.06	0.93	0.84	TC1653151
281	A_51_P334930	1.32	0.66	0.77	0.62	0.84	1810030J14Rik
282	A_51_P333923	0.88	0.93	0.87	0.67	0.84	Tspan1
283	A_52_P177161	0.63	1.02	0.63	1.06	0.83	Pbrm1
284	A_51_P126476	0.68	0.92	0.87	0.84	0.83	Myo1b
285	A_52_P316933	0.68	0.62	0.88	1.10	0.82	Sh3bgrl2
286	A_52_P472936	0.61	1.32	0.61	0.74	0.82	Ythdc1
287	A_52_P136966	0.68	0.75	0.88	0.98	0.82	Ift74
288	A_52_P199776	1.05	0.96	0.64	0.59	0.81	Snhg7
289	A_52_P126266	0.60	1.27	0.64	0.74	0.81	Prkab2
290	A_51_P124784	0.90	0.75	0.69	0.90	0.81	Acpl2
291	A_52_P329256	1.08	0.58	0.81	0.74	0.80	Fxn
292	A_52_P347847	0.65	1.33	0.60	0.62	0.80	AU022252
293	A_51_P221428	1.21	0.66	0.60	0.73	0.80	Cbx6
294	A_51_P129360	0.86	0.85	0.81	0.64	0.79	Pthlh

295	A_51_P500713	0.60	0.64	0.90	0.98	0.78	Dck
296	A_51_P114591	0.74	0.61	0.87	0.91	0.78	1110005A03Rik
297	A_51_P141502	0.85	0.78	0.73	0.70	0.77	Nudt21
298	A_51_P362959	0.72	0.73	0.84	0.71	0.75	Fbxo36
299	A_52_P684798	0.73	1.05	0.60	0.61	0.75	Alms1
300	A_51_P102096	0.66	0.75	0.64	0.93	0.75	Myc
301	A_51_P268673	0.61	0.64	0.74	0.98	0.74	2210016H18Rik
302	A_51_P243623	0.61	0.85	0.67	0.66	0.70	Brms1l
303	A_51_P152211	0.63	0.78	0.60	0.71	0.68	Zfp386
304	A_51_P386304	0.62	0.66	0.75	0.66	0.67	Ccni2
305	A_51_P200819	0.59	0.70	0.63	0.61	0.63	Prdx4
306	A_51_P338961	0.65	0.58	0.61	0.60	0.61	Qser1

In order to determine if Lgr4/5 genes are downstream targets of the Wnt pathway, the behaviour of this gene set was analysed in two complementary scenarios that detect intestinal Wnt target genes. In the first, published scenario[115], deletion of *Apc* results in the immediate upregulation of Wnt pathway target genes. Comparison of the *Lgr4/5* deletion gene set to the microarray data from Sansom, O.J. et al.,[115] showed a significant upregulation of 53% (137 genes) of the *Lgr4/5* gene set, whereas only 5% (12 genes) were significantly downregulated. The remaining 42% also showed a clear tendency to be upregulated (Fig 2, dark ratios). Next, Enrichment Analysis (GSEA) was used to determine whether the gene set is significantly enriched in a certain biological state, such as upon Wnt activation (Subramanian et al, PNAS, 2005). GSEA showed a highly significant enrichment (FDR and p-value <0.0001) of the Lgr4/5 gene set towards the upregulated genes after Apc deletion (Fig 2C). For the second scenario, an *in vitro* crypt organoid culture, which is strictly dependent on the Wnt agonist Rspondin1 (Rspo1)[116], was employed. Rspo-1 was acutely withheld from established small intestinal crypt organoids and performed differential gene expression arraying before, and 1 day after withdrawal (to be deposited at GEO). Rspo-1 withdrawal resulted in the immediate significant downregulation of 38% (166 genes) of the *Lgr4/5* gene set, while only 4% (11 genes) showed the opposite behaviour. The remaining 58% showed a clear tendency towards downregulation (Fig 2, light ratios). GSEA analysis confirmed the highly significant enrichment (FDR and p-value <0.0001) of the *Lgr4/5* gene set towards the downregulated genes after Rspo-1 withdrawal (Fig 2D). Thus, simultaneous deletion of

Lgr4 and *-5* resulted in the immediate downregulation of many genes that are under the control of the Wnt pathway. This suggests a previously unknown role for Lgr proteins in the Wnt signaling pathway.

To address the molecular context in which the Lgr receptors function, a tandem affinity purification mass spectrometric strategy was pursued. Bait proteins were generated by double (Flag-HA)-tagging[117]. As a pilot, double-tagged Frizzled7 (Frz7-FH) were transiently transfected into HEK293T cells. The cells were lysed and immunoprecipitated with an anti-Flag antibody. The immunoprecipitated material was eluted under native conditions with Flag peptide, and re-precipitated with the anti-HA antibody after which mass spectrometric analysis was performed. Significant signatures were detected for Frz7 and, as expected, the Wnt co-receptors LRP5 and LRP6. However, surprisingly, multiple peptides were also detected with high confidence, belonging to endogenous Lgr4 (Fig. 3). Of note, Lgr4 or its family members were not observed using the same protocol in HEK293 cells for approximately 20 unrelated baits. Nor were Frizzleds or LRP5/6 ever observed with these bait proteins.

Stable clones of LS174T colorectal cancer cells were then generated that moderately overexpressed double-tagged versions of Lgr4, Lgr5 or Frizzled5 (Frz5). The Lgr4 bait captured Lgr5 LRP6, Frz5 and Frz7. In four independent experiments conducted with Lgr5 as bait, Frz6 and LRP5 and LRP6 were detected. Using Frz5 as bait, we identified Lgr4, Lgr5, LRP5 and LRP6. These proteins were never observed in non-transfected controls that were run in parallel. These results are summarized in Fig 3. From this set of experiments, it was concluded that Lgr4 and Lgr5 can occur in a physical complex with Frizzleds and LRP5/6.

In an independent set of experiments, the inventors pursued the identification of the Rspo-1 receptor. The four secreted Rspondin proteins, only encoded in vertebrate genomes, activate the canonical Wnt pathway as measured by increases in β-catenin levels and in β-catenin/Tcf reporter assays. They are particularly potent when synergizing with secreted Wnt proteins[118, 119]. Systemic delivery of Rspo-1 in mice leads to a dramatic enhancement of the size and proliferative activity of Wnt-responsive

intestinal crypts[120] and stimulates repair after epithelial injury[121]. While Rspondin3 has recently been shown to utilize syndecans as receptors to mediate non-canonical Wnt signals[122], the nature of Rspondin receptors that drive canonical Wnt signals has remained controversial. Rspo-1 has been reported to be a high affinity ligand of the Wnt co-receptor LRP6[123, 124]. Rspo-1 has also been postulated to bind and block the Kremen protein that down-regulates surface expression of Wnt receptors[125]. In a fourth study, Rspo-1 has been proposed to activate the Wnt pathway by blocking the interaction of the Wnt inhibitor Dkk1 with the LRP6 co-receptor[126].

HEK293T cells are responsive to Wnt3A and Rspondin[127]. Therefore, the inventors decided to use this human kidney cell line in an effort to identify the cognate Rspondin receptor. In order to validate the efficacy of using soluble baits for the discovery of surface receptors, a double (Flag-HA)-tagged version of human Dkk1 (Dkk1-FH) was readily produced at ~1 µg/ml by transient transfection into HEK293T cells. Dkk1 is known to interact with Lrp5/6 and block Wnt signaling[126]. The inventors incubated $2x10^9$ HEK293T cells with the Dkk1-FH conditioned medium on ice. The cells were then washed several times, lysed and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated material was eluted under native conditions with FLAG peptide, and re-precipitated with the anti-HA antibody, after which mass spectrometric analysis was performed. In this analysis, Dkk1 and endogenous LRP5 and LRP6 (Fig 3) were identified, proving that the strategy worked to identify surface receptors.

A double (FLAG-HA)-tagged version of human Rspo-1 (Rspo1-FH) was then produced at ~1 µg/ml in HEK293T cells, the concentration that works optimally in crypt organoid culture[116]. As tested in the β-catenin/TCF reporter TOPFlash reporter assay[128], the protein was functional, potentiating the effect of Wnt3A by ~12-fold (Fig 4). Then $2x10^9$ HEK293 cells were incubated with the Rspo1-FH conditioned medium on ice and subjected them to the above protocol. A dominant protein in terms of peptide identifications in the mass spectrometric analysis was Rspo-1. The only transmembrane protein detected was Lgr4 (Fig 3).

To confirm this interaction, HEK293T cells were transfected transiently with FLAG/HA-tagged versions of Lgr4, Lgr5 or Lgr6, and with tagged Frz5 as control. Transfected cells were incubated with a conditioned medium containing an Rspo1-Fc fusion protein at ~1 µg/ml, washed, lysed and Rspo1-Fc was immunoprecipitated with proteinG beads. Western blotting for the FLAG tag revealed binding of soluble Rspo1-Fc to Lgr4, Lgr5 and Lgr6, but not to Frz5 (Figure 4A). Soluble Rspo1-FH also interacted with the leucine-rich-repeat exodomain of Lgr5 (amino acids 1 to 546) expressed as a human IgG-Fc fusion protein (Lgr5-exo-Fc). This allowed us to perform a binding experiment using Surface Plasmon Resonance array imaging. Anti-FLAG antibody spotted on the sensor chip, readily captured Rspo-1-FH (Fig 4B Left). After a wash, Lgr5-exo-Fc bound to Rspo1-FH (left). After regeneration, a control experiment was performed using a Noggin-Fc fusion protein (Fig 4B, right), which did not bind Rspo1-FH. Both Lgr5-exo-Fc and Noggin-Fc could be captured on anti-human IgG spotted as a control (Fig 4B). The K_D of the Lgr5-exo-Fc / Rspo1-FH interaction was determined at ~3 nM by a 1:1 discrete interaction model at low ligand and low analyte concentration (Scrubber 2.0, BioLogic Software, Australia) (see Fig 6).

Table 3. List of Mass Spectrometry baits that did not yield peptides from Frizzled proteins, Lgr4-6, or Lrp5/6 proteins

betaTrCP1/FBXW1
betaTrCP2/FBXW11
eEF2K
ASB11
KCTD11
KCTD21
RNF43
BHLHE40
UCHL1
USP47
FBXW9

FBXW4
FBXW5
FBXO3
FBXO18
FBXO31
FBXL2
EMI1
E2F3A
E2F3B
PER2

A series of rat monoclonal antibodies was raised against the full-length human Lgr5 protein and described in WO2010/016766[139], which is incorporated herein in its entirety. Table 4 provides an overview of working names for each mAb and the corresponding original sub-clone identities.

Table 4: Working names and subclone IDs for anti-Lgr5 antibodies.

Lgr5 mAb name	**Subclone ID**
Ab1	1D9 A5
Ab4	4D11 F8
Ab5	6C10 D4
Ab6	3A4 G6
Ab7	5A7 F10
Ab9	9G5 E5
Ab10	2B8 A7
Ab11	3B9 D9
Ab12	5C8 G9
Ab13	7B11 G11
Ab14	8F2 B4
Ab16	10C1 C7

All antibodies reacted with Lgr5-exo-Fc and their epitopes were mapped using C-terminal deletion clones as well as human-mouse hybrids of the Lgr5 exodomain. The results are summarized in Fig 4C. Preincubation of Lgr5-exo-Fc with these antibodies

prior to exposure to Rspo1-FH revealed that all antibodies recognizing the region comprising the N-terminus and leucine rich repeat 1 (LRR1) of Lgr5 blocked the Rspo1 interaction, whereas the other antibodies did not (Fig 4D).

To test if Lgr4 constitutes a functional Rspo-1 receptor, *Lgr4* mRNA was removed from HEK293T cells by three independent siRNAs (each of which significantly reduced Lgr4 expression as assessed by Northern blotting; Fig 5A). Subsequently, the cells were transfected with TOPFlash Wnt reporters and their response to exogenously added Wnt3A and Rspo-1 was measured. Wnt3A alone induced a ~25 fold increase in TOPFlash activity. Removal of *Lgr4* had no effect on this response to exogenous Wnt3A (Fig 5B; compare bar 1 to bar 2-5). The combination of Wnt3A with Rspo-1 led to a further ~12 fold increase in TOPFlash activity (bar 6). This effect was greatly diminished in cells from which *Lgr4* was removed (Fig 5B; bars 7, 10 and 14), but could be rescued by transfection of very low amounts (5ng) of *Lgr4* (bars 8, 12 and 16), *Lgr5* (bars 9, 13 and 17) or *Lgr6* expression plasmids (bars 10, 14 and 18). This implied that Lgr4 is dispensable for Wnt3A-mediated input into this pathway, but is necessary for transmitting Rspo-1-mediated input into the canonical Wnt pathway. Thus, Lgr4 is a facultative component of the Wnt receptor complex. Given that Lgr5 and Lgr6 also bind Rspo-1 and can functionally rescue loss of Lgr4, the three family members appear to fulfill similar biochemical roles.

Taken together, these data imply that the members of the Lgr4/5/6 family reside in Frizzled-LRP5/6 complexes and bind soluble Rspondins. Engagement of Lgr proteins by Rspondins triggers downstream canonical Wnt signals through the associated Frizzled-Lrp5/6 complex. This notion is in agreement with the observation that Rspondins, like Wnt proteins, induce LRP6 phosphorylation[124]. The current data do not immediately explain why Rspondin signalling appears dependent on the presence of Wnt proteins. Possibly, Wnt interactions with their cognate Frizzled-Lrp5/6 complexes induce conformational or biochemical changes in the receptor complex that are essential for the subsequent enhancement of signalling activity by Rspondin/Lgr interaction.

The inventors have previously shown that Lgr5 is itself a Wnt target gene and marks stem cells in multiple Wnt-dependent adult stem cell compartments. The current observations provide a molecular explanation for the expression of *Lgr5* and the related gene *Lgr4* by Wnt-dependent stem- and progenitor cells. In intestinal crypts, Lgr4 and Lgr5, incorporated into Frizzled/LRP complexes, allow Rspondins to augment short-range Wnt signals emanating from Paneth cells[128]. Crypt stem cells co-express *Lgr4* and *Lgr5*, while their undifferentiated progenitors only express *Lgr4*. Indeed, we show here that the combined deletion of *Lgr4* and -5 phenocopies loss of Wnt signaling activity. *Lgr4* and *Lgr5* may only differ in their expression pattern, or the signals transmitted through Lgr4 and Lgr5 proteins may be subtly different, such that Lgr5 would provide a stem cell-specific version of Wnt signal potentiation by Rspondins.

Overall, these surprising results demonstrate a previously unknown mechanism for Rsponding signalling through Lgr protein receptors. Unexpectedly, Lgr proteins form part of a Wnt receptor complex (Lgr-Frz-LRP) for activating Wnt/β-signalling. Now that it is known that this receptor complex has two ligands – Wnt and Rspondin – this opens up new opportunities to design Rspondin mimetics, which mimic the role of Rspondin in activating Wnt/β-signalling. Such mimetic compounds could be used alone, or in combination with conventional Wnt agonists, for therapeutic reasons, as well as for tissue engineering and cell culture.

Figures 7-18 further support the findings described in Example 1. For further information refer to De Lau et al. Nature, 476, 293–297, 2011.

MATERIALS AND METHODS FOR EXAMPLE 1

Microarray analysis

Lgr4/5 knock-out mice

Small intestinal crypts were isolated 1 day after induction of deletion from *AHCre Lgr4*$^{fl/fl}$ *Lgr5*$^{fl/fl}$ as well as wt mice by incubation in 2mM EDTA. Intestines were cut open along the length and villi were removed by scraping with a glass slide (Starfrost

microscope slides, 76 x 26 mm, Waldemar Knittel Glasbeabeitungs GmbH, Germany). The intestines were cut into pieces of approx. 3 cm and washed twice with ice cold DPBS (Gibco 14190). The intestinal fragments were then incubated in DPBS (Gibco 14190) supplemented with 5 mM EGTA (Sigma-Aldrich E4378) for 30 minutes incubation at 4 °C. After shaking, the intestinal crypts are in the supernatant. Optional; the intestines were transferred to fresh PBS/EGTA and the procedure was repeated. The supernatant fractions containing the crypts were combined and spun for 5 minutes at 41 g to collect crypts. RNA was isolated from purified crypts using Trizol (Invitrogen, 15596-018) according to the manufacturer's procedures and 1µg of RNA was labeled using Quick Amp Labelling Kit, two colour using Quick Amp Labeling Kit (Agilent, 5190-0444) according to the manufacturer's procedures (Manual: version 5.5 February 2007) with Cy5 and Cy3, respectively; all as described elsewhere[103]. Two separate biological replicates were performed in dye-swap, resulting in 4 individual arrays. Labeling, hybridization, and washing were done according to Agilent guidelines. 825 ng of differentially labeled cRNA product was hybridized to 4 x 44K Agilent Whole Mouse genome dual color arrays (G4122F). Array data were normalized and retrieved using Feature Extraction (V.9.5.3, Agilent Technologies) and data analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Features were flagged, if signal intensities for both the Cy3 and Cy5 channel did not pass the Feature Extraction Filter "Significant and Positive" or "Well above Background". Genes were considered downregulated if 4/4 arrays showed a significant ($p<0.05$) downregulation of <-0.58 (linear -1.5 fold). This resulted in 379 entries, which 307 unique genes (Table 2: Array data will be available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

Rspondin1 withdrawal in mouse intestinal organoids

Crypts were isolated from a wild type mouse small intestine by incubating with 2mM EDTA in PBS for 30 min at 4°C, and subsequently cultured in the crypt culture medium as reported previously[116]. Briefly, isolated crypts were cultured in Matrigel (BD Bioscience) in 24-well plates, and advanced DMEM/F12 medium (Invitrogen) containing EGF, Noggin and Rspo-1 was added after polymerization of Matrigel.

Confluent organoids were split into multiple wells, and were then cultured in crypt culture medium in the presence or absence of Rspo-1. One day after Rspo-1 withdrawal organoids (-RSpo1 organoids) and the control organoids (+Rspo1 organoids) were then collected for RNA extraction and microarray analysis. One µg of RNA from control and Rspo-1 depleted organoids, together with universal mouse reference RNA (Strategene) was labeled using Quick Amp Labelling Kit, two colour (Agilent Technologies) with Cy5 and Cy3 respectively. Samples were hybridized to 4X44K Whole Mouse Genome Microarrays (Agilent, G4122F) according to manufacture's instruction. Microarray signal and background information were retrieved and normalized using Feature Extraction program (V.9.5.3, Agilent Technologies). Samples were considered as well-measured when the fluorescent signals in red channel (Cy5) in either of the samples were greater than 2 fold above the local background. Differences between -Rspo1 organoids and +Rspo1 organoids were calculated by subtracting the ratio for "-RSpo1 organoids vs reference RNA" from "+Rspo1 vs reference RNA". Array data will be available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) upon publication.

Heatmap and Gene Set Enrichment Analysis

Heatmaps were generated using MeV (Multiple Experiment Viewer v.4.3) (Saeed AI, Biotechniques, 2003). Heatmaps were generated using the 306 genes from the Lgr4/5 gene set and plotting the ratios of two different experiments for these genes. Fig2A contains the ratios from [115], where the authors deleted *Apc* in the *AhCre Apc*$^{fl/fl}$ mice and performed microarray analysis 3 days after deletion. Fig2B contains the ratios form the Rspo-1 withdrawal experiment described above. Gene set enrichment analysis (GSEA) implemented with GSEA-P v.2.0 (http://www.broad.mit.edu/gsea) was used to identify significant enrichments of the Lgr4/5 gene set in the two different experimental scenarios mentioned above. All "well-measured" features (n=20844) for the Rspo-1 experiment from the Agilent arrays and all features with a ratio (n=45101) for the *Apc* arrays from the Affymetrix platform were used for the Ranked gene list in GSEA.

Mass Spectrometric analysis. The samples were subjected to SDS-PAGE followed by Coomassie blue staining. The gel lane was sliced into 24 equal sections and subjected to

digestion. In brief, protein reduction and alkylation was performed with DTT (60 °C, 1 hour) and Iodoacetamide (dark, RT, 30min), respectively. Digestion was performed with trypsin over night at 37 °C. Peptides were extracted with 10 % FA[130].

The extracted peptides were subjected to nanoscale liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) analysis, performed on an Agilent 1200 HPLC system (Agilent technologies) connected to an LTQ Orbitrap Velos (ThermoFisher, Waltham, MA)[131]. The nanoLC was equipped with a 20 mm x 100 µm i.d. trap column and a 400 mm x 50 µm i.d. analytical column (Reprosil C18, Dr Maisch, Ammerbuch-Entringen, Germany). Trapping was performed at a flow of 5 µL/min for 10 min and the fractions were eluted using a 45 min linear gradient from 0 to 40 % solvent B (0.1 M acetic acid in 80 % ACN (v/v), in which solvent A was 0.1 M acetic acid), 40 to 100 % solvent B in 2 min and 100 % B for 2.5 min. The analytical flow rate was 100 nL/min and the column effluent was directly introduced into the ESI source of the MS using a standard coated fused silica emitter (New Objective, Woburn, MA, USA) (o.d. 360 µm, tip i.d. 10 µm) biased to 1.7 kV. The mass spectrometer was operated in positive ion mode and in data-dependent mode to automatically switch between MS and MSMS. The five most intense ions in the survey scan were fragmented in the linear ion trap using collision induced dissociation[132]. The target ion setting was 5e5 for the Orbitrap, with a maximum fill-time of 250 ms. Fragment ion spectra were acquired in the LTQ with an AGC value of 5e3 and a max injection time of 100 ms.

Protein Identification: Raw MS data were converted to peak lists using MaxQuant version 1.0.13.13[133]. Spectra were searched against the IPI (International Protein Index) Human database version 3.37 (69,164 sequences; 29,064,824 residues) using Mascot search engine (version 2.3.02; www.matrixscience.com), with trypsin set as enzyme. The database search was made with the following parameters set to consider a peptide tolerance of ± 15 ppm, a fragment tolerance of ± 0.5 Da, allowing 2 missed cleavages, carbamidomethyl (C) as fixed modification, oxidation (M) and protein N-terminal acetylation as variable modifications.

Plasmon Surface resonance. A pre-activated ester sensor chip (IBIS Technologies, Enschede, The Netherlands) was spotted using a Continuous Flow Microspotter (Wasatch Microfluidics (Salt Lake City, Utah, US). In total, 32 spots were created with both mouse antiFLAG, goat antihuman IgG in 8x serial dilution (start concentration is 500 ug/ml and 100 ug/ml respectively) in 10 mM MES buffer pH 5.5. Control (reference) spots contained HSA, antiHSA and MES buffer. After preparing the sensorchip, the slider was positioned in the instrument IBIS MX96 (IBIS Technologies, Enschede, The Netherlands) for label free Surface Plasmon Resonance (SPR) array analysis. The instrument enables multiplex interactions up to 96-plex using scanning imaging optics for automated calculation of the SPR-dip shifts of all Region of Interests simultaneously. The signal to noise ratio of the instrument which reflects the limit of detection is better than 1 RU corresponding to 1 picogram protein per square mm. In the IBIS MX96, back and forth mixing is applied enabling minimal use of sample while the length of the exposure of the sample to the microarray is unlimited and not affected by the volume of e.g. an injection loop. A two step interaction process was carried out and the multiplex interaction event to all spots of the array was recorded simultaneously. In this way, chip to chip and sample to sample variations can be excluded, while positive- and negative controls and referencing can be applied instantly. A microscope image of the sensor chip (not shown here) can reveal any irregularities, inhomogeneities of the spots and/or disturbing air-bubbles. First 70 microliter of RSPO1-FH was injected followed by dissociation and second injection of 70 µl LGR5-Fc. After regeneration with acid buffer (Gly-HCl, 10 mM, pH=2.0) for 120 seconds, the control experiment was capture of RSPO1-FH followed by injection of Noggin-Fc (results see figure 4B). For affinity constant determination first RSPO1-FH was captured on the anti-Flag spot until saturation of the spot followed by injection of LGR5-Fc (8 ug/ml) The RSPO1 was not immobilized directly on the chip because RSPO1 did not survive the acid regeneration process (data not shown). Capturing of RSPO1-FH was possible on the anti-Flag antibody spot from growth medium. Figure 1 is an overlay plot of three interaction series of first RSPO1-FH (2 minutes association was sufficient to reach saturation) and three different concentrations of LGR5-Fc (Mw 176 kD) corresponding to 45 nM, 23 nM and 11 nM injections. Referencing was carried out by subtraction of the anti-FLag spot

signal with the signal coming from a HSA loaded spot in SPRint software (IBIS Technologies, Enschede, The Netherlands) for compensating bulk refractive index shifts e.g. as a result of growth medium compounds. The affinity constant was calculated using a discrete 1:1 interaction model using global fitting (Scrubber 2, BioLogic Software Pty Ltd, Australia). In Figure 6, the residual plot of the experimental curve minus fit values is shown and although it cannot be revealed that the interaction process is according to a discrete 1:1 interaction model the affinity constant was calculated to be ~3 nM. It was not necessary to add an extra fit parameter for mass transport limitation compensation, due to a high back and forth mixing condition in the IBIS MX96 flow cell.

Large -Scale Purification

Transfection and Harvest: cells were cultured as previously described in a number of 15 cm plates according to the number of cells required. Typically, 40 plates were used for 4-5 tandem affinity purifications (TAPs): 40x 15 cm plates for Lgr4&5 TAP (LS174T cells); 40x 15cm plates for Frz5 TAP (LS174T cells); 80x 15 cm plates for hRspo1 TAP (HEK293T cells); 80-15 cm plates for DKK TAP (HEK293T cells). 40x15 cm plates of 4 or 5 TAP's were grown to 80% full. The cells were collected into 50 ml conical flasks and washed twice with PBS at room temperature (RT). Cells were spun for 5 min. at 1200 rpm (Centrifuge 5810R, Eppendorf). The supernatant was aspirated and the cells were frozen in dry ice at -80 °C. All further steps are carried out at 4 °C.

Lysis: the lysis buffer was prepared with (LBI) and without (LB) inhibitors in a clean bottle (LBI in 0.5% NP40). The pellet was resuspended in 40 ml of LBI and incubated on ice for 1 hour. The tube was gently inverted every 6-7 min without vortexing. Following incubation, the tube was spun for 30 min at 20,000 rpm at 4°C (Centrifuge Avanti J-30I, Rotor JA 25.50, Beckman Coulter). The supernatant, containing cell lysate free from cell membranes, was transferred into a new tube and kept on ice.

Filtration: approximately 5x 0.22uM filters (Whatmann FP 30/0,45 CA-S Ref. No. 10462100) were prepared along with a 20 ml syringe for each purification. The plunger removed, the filter attached, the lysate loaded (not to the top), the plunger inserted and the lysate filtered into a new tube. Typically, about 2-3 filters were needed for each

purification. This step removes other debris from the cell lysate and the volume of the filtered lysate will be slightly lower than the initial volume.

Preclearing: 850 µl / 2 Immunoprecipitations (IPs) were transferred into a 15 ml tube using a cut blue tip. 10 ml of LBI was added and the tube was inverted 6-7 times followed be spinning for 2 min at 1200 rpm. The supernatant was aspirated and a LBI was added in a 1:1 ratio of beads:LBI. It was mixed to resuspend well. 400µl slurry was transferred into each of the two cell lysate tubes and the tubes were incubated for 60 min. on a wheel (Test-tube rotator Snijder 34528) at 4°C. To remove the protein-G-agarose from the precleared lysate the tubes were spun at 3000 rpm 2' (Centrifuge 5810R, Eppendorf) and the supernatant was collected. Filter sample with a 0.22 µM filter.

FLAG IP: a volume (500 µl/2 IP's) of M2 FLAG resin (anti-FLAG M2 affinity gel, Sigma A2220-5ML) was transferred to a 15 ml conical tube using a cut blue tip. 10 ml of LBI was added and gently mixed by inverting the tube 6-7 times followed by spinning for 2 min at 1200 rpm. The supernatant was aspirated and resuspended in the resin at a 1:1 ratio of beads:LBI. 250 µl slurry of M2 FLAG resin was added to each sample. This was incubated for 2 hours on a wheel in a cold room. The product of these steps was IPs of anti-FLAG bound to FLAG portion of double-tagged proteins (FLAG-HA-Tagged).

Washes: samples were spun at 1200 rpm for 2 min and the supernatant removed by aspiration. The wash was achieved by adding 10 ml of LBI and mixing by inverting the tube gently 6-7 times and spinning at 1200 rpm for 2 min. Four more washes were performed in 1.5 ml eppendorf tubes using 1 ml of LBI each time.

FLAG Elution (performed at room temperature): after the last wash, the supernatant was aspirated (not completely) and 1.2 ml of the diluted 3XFLAG peptide (100ug/ml) was added per IP. A dilution of 3XFLAG peptide (Sigma F4799-4MG) in LBI: [stock] = 2mg/ml; [final] = 100 µg/ml. 125 µl stock of 3XFLAG peptide was purified in 2.5 ml of LBI (changing the volume where required). This was followed by incubation on the wheel for 20 min at room temperature. The resulting product of these steps was FLAG-HA-tagged protein without anti-FLAG; antiFLAG is now bound to FLAG-peptide which

was added in excess; the complexed beads anti-FLAG-FLAG peptide were then removed by the centrifuge step in the HA IP steps below.

HA IP (all steps at 4 °C): the sample was spun at 3000 rpm twice, then purified on a Millipore Millex GV4 0.22um filter. 120ul/IP slurry of HA resin (anti-HA affinity matrix Rat monoclonal, ref 11815016001, Roche) was transferred into 15ml tube using a cut yellow tip. 10 ml of LBI was added and mixed by gently inverting the tube for 6-7 times. The resin was spun down 1200rpm for 2 min and the supernatant removed by aspiration. The resin was resuspended to a ratio of 1:1 beads:LBI. The eluate was added to the HA resin and the tube containing the HA IP was incubate on the wheel for 2 hours at 4°C. These steps provided immunoprecipitates comprising of anti-HA bound to HA portion of double-tagged proteins (FLAG-HA-Tagged).

Washes: after removing the supernatant, two washes (1 ml each) were carried out in LBI, gently inverting the tube 6-7 times. This was followed by spinning at 1200 rpm for 2 min and 3 further 1 ml washes in LB.

HA Elution: the samples were resuspended in 150 µl of LB and transferred into one spin column filter (Biorad micro biospin column catno. 732-6204) and collection tube. Each sample was spun at 1400 rpm for 1 min. The filter containing the beads was transferred onto a new clean eppendorf tube and 50 µl of 2X Laemmli sample buffer (Sigma, S3401-10VL) were added to the beads. The tube was incubated for 1 min and spun at 1400 rpm for 1 min. This was repeated for additional sequential elutions. The samples were then frozen at -80°. The eluted product was a double tagged protein without anti-HA. (Laemmli sample buffer denatures the anti-HA antibody and therefore the anti-HA is removed from the FLAG-HA-tagged protein. The remaining bait protein plus its captured proteins is what is left.)

Silver Staining with SilverQuest™ Silver Staining Kit (Cat # LC6070, Invitrogen): 10 µl of each sample (HA elution) were loaded on a 1 mm 4-12% NuPAGE Bis-Tris Gel (cat # NP0321BOX, Invitrogen). 5 µl of the FLAG elution (total sample 200 µl) were loaded followed by 2 µl of marker and 5 µl of 2X sample buffer. The BASIC protocol of

the SilverQuest™ kit was followed. The steps were carried out in a 50 ml volume using 50 ml conical tubes (instead of 100 ml).

Lysis buffer (LB):

50 mM Tris HCl pH 7.4
150 mM NaCl
1 mM $MgCl_2$
10 % Glycerol
0.5 % NP40
1 mM CaCl2
H_2O (MilliQ)

Lysis buffer + inhibitors (LBI):

50 ml LB + 1 tablet protease inhibitor cocktail (Complete EDTA-free Protease Inhibitor Cocktail tablet; Cat no 11873580001, Roche Applied Science)

Example 2: The Furin domain of Rspondin is sufficient for binding and Rspondin activity

The inventors generated truncated Rspondin fragments including: (i) deletion of the basic amino acid domain (hRSP4 daa) (SEQ ID NO: 139) and (ii) deletion of the thrombospondin domain and basic domain (hRSPO4 dtht+ aa) (SEQ ID NO: 140). These truncated Rspondin proteins, and Wild type full-length Rspondin (hRSPO4) as a control, were used to precipitate hLgr5 (Fc-tagged) and DKK (Flag-tagged). It was found that DKK does not bind to hRSPO4 or any of the fragments of hRSPO4. By contrast, hLgr5 coimmunoprecipitated with full-length Rspondin, and also with both Rspondin fragments. The fragment hRSPO4 dtht+aa represents the Furin domain of Rspondin4 (both the thrombospondin and basic domains have been deleted) (see Figure 19). Therefore, the results show that the Furin domain is sufficient for binding to Lgr5.

The inventors have also shown that an anti-Rspondin 3 monoclonal antibody, which binds to epitopes in the Furin domain of Rspondin 3, blocks the Wnt-enhancing activity

of Rspondin (for example, see Figure 21). HEK293T cells were transfected with Wnt pathway-specific (TOP) luciferase reporter in combination with a TK driven Renilla reporter. After 24 hrs cells were incubated for 24 hrs with control conditioned medium (CM), Wnt3a CM, or combinations of Wnt3a + Rspondin-CM. The relative volume proportion (%) of Rspondin CM is indicated in Figure 21. A rat monoclonal antibody (R&D systems, clone 400403), recognizing the furin domain of both human and mouse Rspondin3, was added to a final concentration of 2.5 µg/ml. Wnt pathway activity was quantitated by comparing averages of triplicate Renilla-normalized TOP luciferase counts of cells receiving stimuli, to un-stimulated cells. Figure 21 shows that the TOP luciferase counts (i.e. Wnt pathway activity) is lower when the anti-Rspondin-3 antibody is added to the assay. The effect is greater when a lower proportion of Rspondin is used in the assay. Therefore, the inventors conclude that the Furin domain is sufficient for binding and also necessary for the activity of Rspondin.

In another experiment, the inventors tested the activity (in terms of Wnt enhancement) of the Rspondin fragments (as described above) in a TCF reporter assay. Figure 22 shows the results of this assay. Columns 19-20 show luciferase counts for the furin domain fragment of Rspondin (SEQ ID NO: 140) and demonstrate that the furin domain fragment activates the Wnt pathway to a greater extent than full length Rspondin or the Rspondin fragment comprising thrombospondin and furin domains (SEQ ID NO: 139). This experiment shows that the furin domain is not only necessary but also sufficient for mimicking full-length Rspondin Wnt activation. Furthermore, it suggests that the furin domain fragments are more effective at enhancing the Wnt pathway than full-length Rspondin. Therefore, the furin domain fragment is an example of a surprisingly potent agonist of the invention.

Materials and methods for coimmunoprecipitation experiments (Figure 19):

M2-beads, αFLAG staining

1. hRSPO4-WT-TAP (step 2: + hRSPO4-WT-TAP)
2. hRSPO4-dAA-TAP (step 2: + hRSPO4-dAA-TAP)

3. hRSPO4-dTHR+AA-TAP (step 2: hRSPO4-dTHR+AA-TAP)

M2-beads, αFc staining

1. hRSPO4-WT-TAP (step 2: + Dkk1-Fc)
2. hRSPO4-dAA-TAP (step 2: + Dkk1-Fc)
3. hRSPO4-dTHR+AA-TAP (step 2: + Dkk1-Fc)
4. Dkk1-Fc (step 2: + Dkk1-Fc)
5. hRSPO4-WT-TAP (step 2: +LGR5exo-Fc)
6. hRSPO4-dAA-TAP (step 2: +LGR5exo-Fc)
7. hRSPO4-dTHR+AA-TAP (step 2: +LGR5exo-Fc)
8. LGR5exo-Fc (step 2: +LGR5exo-Fc)

All actions performed on ice or at 4˚C

- Cut the top off a blue pipet tip and pipet the appropriate (total) amount of beads needed into a 15ml tube (11 x 30ul = 360 ul)
- Wash the beads with 10 ml lysis buffer (LB)
- Spin down at 1200 rpm for 2 min at 4˚C
- Add 11 ml of LB and mix well
- Add 1 ml of the mixture to each Eppendorf tube
- Spin down for 2 min at 1200 rpm
- Remove the LB
- Incubate overnight with 2 ml of step 1 CM at 4˚C on a spinning wheel
- Wash twice with 2 ml of LB (spin down at 1200 rpm for 2 min at 4˚C)
- Transfer to a 1.5 ml Eppendorf tube
- Add 1.5 ml of LGR5-Fc CM and incubated at 4˚C on a spinning wheel for four hours
- Wash four times with 1 ml of LB
- Carefully remove all LB
- Add 40 ul of 2x sample buffer was and mix well
- Heatshock for 3 min at 70˚C
- Spin down at 1200 rpm, 4˚C for 2 min
- Load 30 µl on a 10% gel

- Load empty lanes with 20 µl of 2x sample buffer
- Load 8 µl of a PageRuler™ prestained protein ladder
- Run gel at 80 V through the stacking and then at 100 V through the separation gel
- Blot overnight at 4° at 100 mA
- Block the blots with a 10% milk in TBS solution for 1h
- Stain the blots with 1:2000 αFLAG or 1:5000 αhIgG respectively in 10% milk in TBST
- Wash the blots for 45 min with TBST, refresh 5 times

Example 3: 1D9 is an agonist of Lgr5

Several known anti-Lgr5 antibodies were tested for agonistic activity towards Lgr5 using the TCF reporter assay. HEK293T cells stably transfected with LGR5 are transfected with the TOP reporter and a TK driven Renilla reporter constructs. After 24 hours the cells were stimulated with: 1) Wnt3A condition medium (Produced in L-Cells and added at 1/3 of the total medium volume), 2) RSpo1 His tagged purified on a nickel column from 293T cells added at 1ug/ml, 3) Anti LGR5 Rat monoclonal antibodies 1D9, 4D11, 8F2 prot A purified at 1ug/ml. Luciferase activity was determined 24h after stimulation.

The graph in figure 20A shows that luciferase count is low in the absence of Rspo1 (less than 20.0), except for where antibody 1D9 is included in the assay. When 1D9 is used in the absence of Rspo1, luciferase activity of approximately 110 is observed, which is similar to the luciferase activity of 100, which is observed when Rspo1 is used in place of 1D9. When both 1D9 and Rspo1 are used together in the assay, Wnt expression is even greater (represented by the luciferase counts of approximately 145). Therefore, 1D9 is an agonist of Lgr5 that mimics the activity of Rspo1.

HEK293T cells were seeded into 96-well plates in DMEM/10% FCS at a density of 10^4cells/well and in triplicate transfected (PEI: Polyethylenimine, linear, MW-25,000) with 10 ng TOP or FOP luciferase (Ref 1), 1 ng TK Renilla, 10 ng of pcDNA-based hLgr5-Flag, and 80 ng empty vector DNA. After 24 hrs, medium was replaced for 50% fresh DMEM/10% FCS, and 50% Wnt3a-conditioned medium or control conditioned medium. At the same time point, purified human Rspondin1 (stock solution of 100

µg/ml in PBS) was added at a final concentration of 1 µg/ml and ProtA-purified (stock solution of 1mg/ml in PBS) Lgr5-specific antibodies. At 72 hrs results were measured using a dual luciferase assay kit (Promega USA).

The graph in figure 20B shows that luciferase count is low in the absence of Rspo1 (approximately 20.0 – see column 7) compared to luciferase count with Rspo1 and Wnt3a (approximately 170 counts – see column 13). Antibody 1D9 in part compensates for the absence of Rspo1 as is shown in columns 19, 25 and 31. The greater the concentration of antibody 1D9 the greater the luciferase counts, demonstrating that antibody 1D9 is acting as an agonist of the Wnt pathway. Antibody 8F2 was did not show any significant increase in Wnt activity compared to Wnt3a in the absence of Rspondin (see columns 7, 37 and 43).

1D9 binds to the hinge region of Lgr5, more specifically the CRL region represented by SEQ ID NO: 57 (see also figure 4C).

Example 4: Multi-targeting agonists

The inventors generated a synthetic fusion protein, by methods well known in the art, comprising an anti-Epcam antibody linked to an Rspondin1 furin domain fragment (SEQ ID NO: 141 represents the sequence of the full Rspondin1 furin domain fragment used including the flexible linker peptide; SEQ ID NO: 142 represents the sequence of the flexible linker peptide alone; and SEQ ID NO: 143 represents the sequence of the furin repeat region in the fusion protein in the Rspondin furin domain fragment). Epcam (also referred to as TACSTD1 or CD326) is is an epithelial cell adhesion molecule which is expressed on most epithelial cells. Epcam is also expressed on the basolateral membrane of the intestine (it faces towards the interstitium, and away from the lumen). The inventors have discovered that Lgr proteins also reside on the basolateral side of the membrane.

By linking the furin domain fragment (an agonist of Lgr proteins – see example 2) to an anti-Epcam antibody, the inventors targeted the furin domain fragment to epithelial cells, and more specifically, to the basolateral side of the epithelial cells, thus generating a

bispecific multi-targeting agonist (targeted to and specific for both Lgr and Epcam). To test the Rspondin-mimicking activity of this multi-targeting agonist, the inventors grew mouse colon organoids in Sato medium, which is known to require Rspondin for organoid growth (see Sato et al., Nature 459, 262-5, 2009). The inventors then replaced the Rspondin with the multi-targeting agonist to observe whether the agonist could rescue growth of the organoids in an Rspondin-depleted medium. It was found that, not only could the mouse organoids grow to an equivalent size in the presence of the multi-targeting agonist, but also that the multi-targeting agonist had a similar effect to Rspondin at a 50x lower concentration (see Figure 24). This demonstrates that the anti-Epcam antibody effectively targets the agonist to epithelial cells and mimics the activity of Rspondin binding to an Lgr protein and enhancing the Wnt pathway, as is required for organoid growth. The fact that the multi-targeting agonist can be used at 50x lower concentrations than Rspondin gives an indication of possible dosage requirements (both for use in therapy and for use in culture media) and also indicates that it could be suitable for use in therapy because lower doses combined with targeted activity would result in fewer unwanted side effects in the patient.

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Notification of completion of the procedure of an original deposit or of a new deposit with another International Depositary Authority

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This International Depositary Authority declares that it accepts the microorganism with the following identification reference,

Identification reference given to the microorganism by the depositor:

1D9 A5

Accession number given by the International Depositary Authority:

LMBP 6961CB

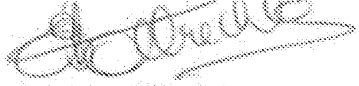
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CLAIMS

1. An agonist of the Wnt pathway which mimics the activity of an Rspondin protein binding to at least one Lgr protein.

2. The agonist of claim 1 wherein the Rspondin protein is Rspondin 1, Rspondin 2, Rspondin 3 or Rspondin 4.

3. The agonist of claim 1 or claim 2, which is not antibody 1D9 and/or does not consist of an Rspondin Furin Domain.

4. The agonist of any one of claims 1 to 3, wherein the at least one Lgr protein is Lgr4, Lgr5 and/or Lgr6.

5. The agonist of any one of claims 1 to 4, wherein the at least one Lgr protein is in a complex with at least one Frizzled receptor and at least one LRP co-receptor.

6. The agonist of claim 5, wherein the Frizzled receptor is at least one of Frizzled 1 to Frizzled 10.

7. The agonist of claim 6, wherein the Frizzled receptor is at least one of Frizzled 5, Frizzled 6 and Frizzled 7.

8. The agonist of any one of claims 5 to 7, wherein the LRP co-receptor is at least one of LRP5 and LRP6.

9. The agonist of any one of claims 5 to 8, wherein the complex further comprises a Wnt protein.

10. The agonist of claim 9, wherein the Wnt protein is Wnt3a.

11. The agonist of claim 9 and 10, wherein the complex further comprises (i) at least one of Lgr4, Lgr5 and Lgr6 and (ii) at least one of Frizzled 5 or Frizzled 6 or Frizzled 7 and (iii) at least one of LRP5 or LRP6.

12. The agonist of any one of the preceding claims, which binds to at least one Lgr protein.

13. The agonist of claim 12, which binds specifically to Lgr4, Lgr5 or Lgr6.

14. The agonist of claim 12, which binds to the extracellular parts, for example one or more extracellular part, or transmembrane regions, for example one or more transmembrane region, of the Lgr protein.

15. The agonist of claim 14, wherein the extracellular parts of the Lgr protein comprises the N-terminal region and/or any of the 17 leucine rich repeats, and/or the CRL region and/or any of the 3 exodomain sequences.

16. The agonist of claim 14 or claim 15, which binds to an epitope within SEQ ID NOs: 2-21, 23-25, 27-29, 31-33, 35-37, 39-58, 60-62, 64-66, 68-70, 72-74, 76-95, 97-99, 101-103, 105-107, 109-111 in the Lgr4, Lgr5 or Lgr6 polypeptide sequence.

17. The agonist of any one of the preceding claims, which binds to a region consisting of or comprising the CRL region of Lgr4, Lgr5 and/or Lgr6, wherein the CRL region is represented by SEQ ID NOs: 20, 57 and/or 94, respectively.

18. The agonist of claim 12, which binds to the Rspondin binding site of the Lgr protein.

19. The agonist of any one of claims 12 to 18, which competes for Rspondin binding.

20. The agonist of any one of claims 12 to 18, which binds to the Lgr protein with a greater affinity and/or avidity than that of Rspondin.

21. The agonist of any one of claims 12 to 18, which binds to the Lgr protein with a Kd equal to or less than about 10^{-7}M, 10^{-8}M, 10^{-9}M, 10^{-10}M, 10^{-11}M, 10^{-12}M, 10^{-13}M or more.

22. The agonist of any one of the preceding claims, which is a polypeptide, a peptidomimetic, an aptamer or a small molecule.

23. The agonist of any one of the preceding claims, which is a fragment or derivative of Rspondin.

24. The agonist of claim 23, wherein the fragment or derivative of Rspondin comprises or consists of an Rspondin Furin domain, for example selected from SEQ ID NOs: 116, 117, 118, 119, 140 or 143.

25. The agonist of any one of the preceding claims, which is an antibody or fragment thereof.

26. The agonist of any one of the preceding claims, which is free of antagonistic activity.

27. The agonist of any one of the preceding claims, which enhances beta-catenin signalling by a factor of 2x, 5x, 10x, 100x, 1000x, 10000x or more as compared to the activity in the absence of the compound.

28. The agonist of claim 27, which enhances beta-catenin signalling by a factor of 2x, 5x, 10x, 100x, 1000x, 10000x or more as measured in terms of one or more of: Wnt/β-catenin target gene expression, TCF reporter gene expression, beta-catenin stabilization, LRP phosphorylation, and/or Axin translocation from cytoplasm to cell membrane and binding to LRP.

29. The agonist of claim 28, wherein the enhanced beta-catenin signalling is measured in the TOPFlash assay.

30. The agonist of any one of claims 1 to 29, wherein the agonist is antibody 1D9.

31. The agonist of any one of claims 12 to 30, which additionally binds to at least one further target polypeptide or other target molecule.

32. A multi-targeting compound, comprising at least one portion with Rspondin-mimicking activity, and at least one other portion that binds to a further target polypeptide or other target molecule.

33. The multi-targeting compound of claim 32, wherein the at least one portion with Rspondin-mimicking activity comprises:

a) an Rspondin protein; or

b) an agonist according to any one of claims 1 to 31.

34. The multi-targeting compound of claim 33, wherein the Rspondin protein comprises or consists of SEQ ID NO: 112, 113, 114 or 115.

35. The agonist of claim 23 or 24 or a multi-targeting compound of claim 33, wherein the agonist is an Rspondin protein fragment comprising or consisting of an amino acid portion of any one of SEQ ID NOs: 112, 113, 114 or 115 which is more than 50, 100, 150 or 200 consecutive amino acids in length.

36. The agonist of any one of claims 23, 24 or 35 or the multi-targeting compound of claim 33 or 35, wherein the Rspondin fragment comprises or consists of an Rspondin Furin domain, wherein preferably, the Rspondin Furin domain has more than 70, 80, 90, 95%, 98% or 99% identity to SEQ ID NOs: 116, 117, 118, 119, 140 or 143, or is 100% identical thereto.

37. The multi-targeting compound of claim 32 or claim 33, wherein the at least one portion with Rspondin-mimicking activity is an antibody that is specific for one of or more than one of Lgr5, Lgr4 and/or Lgr6.

38. The multi-targeting compound of claim 37, wherein the antibody is antibody 1D9.

39. The multi-targeting compound of any one of claims 32 to 38, wherein said multi-targeting compound is a conjugate or fusion protein.

40. The multi-targeting compound of any one of claims 32 to 39, wherein the at least one other portion that binds to a further polypeptide or other target molecule is a polypeptide, a peptidomimetic, an antibody or a fragment thereof, an aptamer or a small molecule.

41. The multi-targeting compound of any one of claims 32 to 39, wherein the at least one other portion that binds to a further polypeptide or other target molecule binds to a basolateral polypeptide or target molecule on the cell or tissue of interest.

42. The agonist of claim 31 or the multi-targeting compound of any one of claims 32 to 41, wherein said further target polypeptide or other target molecule is selected from an Lgr protein, a Frizzled receptor, or a cell- or tissue-specific marker.

43. The agonist or the multi-targeting compound of claim 42, wherein the tissue-specific marker is specific to liver, pancreas, small-intestine, colon, kidney, heart, lung or hair follicle.

44. The agonist or the multi-targeting compound of claim 43, wherein the cell- or tissue-specific marker is selected from the group consisting of Epcam, CA19, A33, L-cadherin, and dipeptidyl peptidase V.

45. The multi-targeting compound of any one of claims 40 to 44, wherein the at least one other portion that binds to a further polypeptide or other target molecule is an anti-Epcam antibody or fragment thereof conjugated or fused to a furin domain fragment comprising or consisting of the sequence in SEQ ID NO: 141 or SEQ ID NO: 143.

46. The agonist or the multi-targeting compound claim 43, wherein the tissue-specific marker is a cell-surface liver-specific marker.

47. The agonist of claim 31 or the multi-targeting compound of any one of claims 32 to 46, which is a bispecific antibody, including but not limited to BiTEs or Tandabs.

48. A composition comprising (a) one or more agonists or multi-targeting compounds according to any one of the preceding claims, and (b) a suitable and/or pharmaceutically acceptable carrier or diluent.

49. The composition according to claim 48, wherein the one or more agonist may be any combination of agonists that target Lgr4, Lgr5 or Lgr6.

50. The composition according to claim 49, wherein the combination of agonists comprises an Lgr4 antibody and an Lgr5 antibody.

51. The composition of any one of claims 48 to 50, which optionally further comprises an effective amount of at least one compound or protein selected from at least one of: an anti-infective drug, a cardiovascular (CV) system drug, a central nervous system (CNS) drug, an autonomic nervous system (ANS) drug, a respiratory tract drug, a gastrointestinal (GI) tract drug, a hormonal drug, a drug for fluid or electrolyte balance, a hematologic drug, an antineoplastic, an immunomodulation drug, an ophthalmic, otic or nasal drug, a topical drug, or a nutritional drug.

52. An agonist or multi-targeting compound according to any one of claims 1 to 47 or a composition according to any one of claims 48 to 51 for use in therapy.

53. An agonist, multi-targeting compound or composition according to claim 52 for use in treating patients with tissue loss or damage due to aging or pathological conditions and/or for use in tissue regeneration.

54. A method of treating a patient suffering from tissue loss or damage or damage due to aging or pathological conditions comprising administering an agonist or multi-targeting compound according to any one of claims 1 to 47 or a composition according to any one of claims 48 to 51 to said patient.

55. The agonist, multi-targeting compound or composition according to claim 53 or the method according to claim 54, wherein the cause of tissue loss or damage includes but is not limited to or is selected from the list consisting of: radiation/chemotherapy, mucositis, IBD, short bowel syndrome, hereditary bowel disorders, celiac disease, metabolic diseases, hereditary syndromes, (viral) infections (hepB/C), toxic states, alcoholic liver, fatty liver, cirrhosis, infections, pernicious anemia, ulceration, diabetes, destruction of islet cells, loss of bone mass (osteoporosis), loss of functional skin, loss of hair, loss of functional lung tissue, loss of kidney tissue (for instance acute tubulus necrosis), and loss of sensory cells in the inner ear.

56. A method for enhancing the proliferation of cells comprising supplying an agonist, multi-targeting compound or composition according to any one of claims 1 to 53 or 55 to said cells.

57. The method according to claim 56, comprising supplying an antibody to said cells, wherein optionally the antibody is antibody 1D9.

58. The method according to claim 56, comprising supplying a multi-targeting compound to said cells, wherein optionally the multi-targeting compound comprises or consists of an anti-Epcam antibody linked to Rspondin1-4 or a Furin domain fragment according to any one of claims 34-36 or 45.

59. The method of claim 58 further comprising supplying the cells with a growth factor.

60. A method for tissue regeneration of damaged tissue comprising administering an agonist, multi-targeting compound or composition according to any one of claims 1 to 53 or 55 to said damaged tissue.

61. The method of any one of claims 56 to 60, wherein the method is carried out *in vivo*, *ex vivo*, or *in vitro*.

62. A method for identifying an agonist of the Wnt pathway, said method comprising:

a) contacting a complex comprising at least one Lgr protein, at least one Frizzled receptor and at least one LRP protein with a candidate compound in the presence of a Wnt protein; and

b) determining the level of Wnt/beta catenin signalling

wherein an increase in the level of Wnt/beta catenin indicates that the candidate compound is an agonist of the Wnt pathway.

63. A bispecific compound, optionally a bi-specific antibody, which binds to both Lgr4 and Lgr5 and inhibits beta-catenin signalling.

64. A method of inhibiting beta-catenin signalling comprising administering the bi-specific compound of claim 63.

65. The method of claim 64, wherein the method is conducted *in vitro*, *in vivo* or *ex vivo*.

66. The method of claim 64, wherein the method is conducted *in vivo* and the compound is administered to a patient to treat cancer.

67. A combination of two or all of i) an inhibitor of Lgr5, ii) an inhibitor of Lgr4 and iii) an inhibitor of Lgr6 for use in treating cancer wherein said two or all of i) an inhibitor of Lgr5, ii) an inhibitor of Lgr4 and iii) an inhibitor of Lgr6 are for sequential, simultaneous or separate administration.

68. A method of treating cancer comprising administering two or all of i) an inhibitor of Lgr5, ii) an inhibitor of Lgr4 and iii) an inhibitor of Lgr6 wherein said two or all of i) an

inhibitor of Lgr5, ii) an inhibitor of Lgr4 and iii) an inhibitor of Lgr6 are administered sequentially, simultaneously or separately.

69. A cell culture medium comprising an agonist or multi-targeting compound according to any one of claims 1 to 47.

70. An organoid obtained using the method of any one of claims 56 to 59 and/or the cell culture medium of claim 69.

FIG. 1(a)

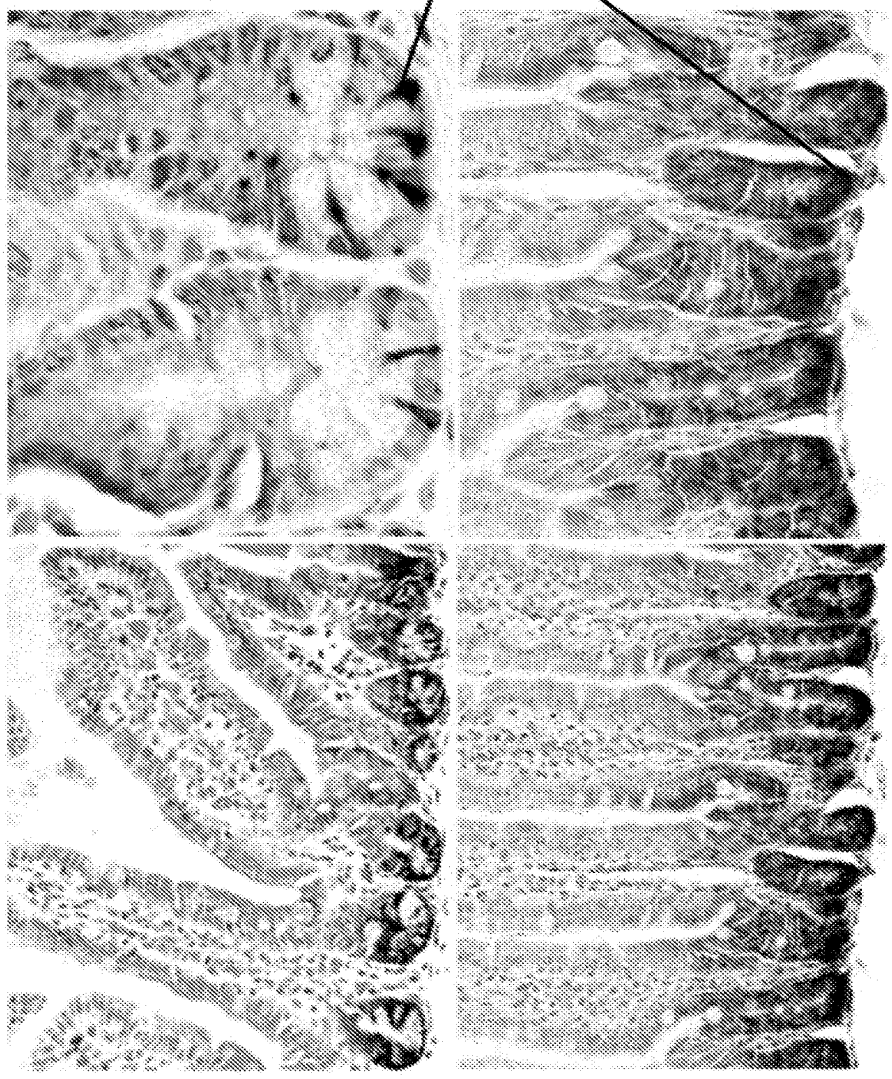
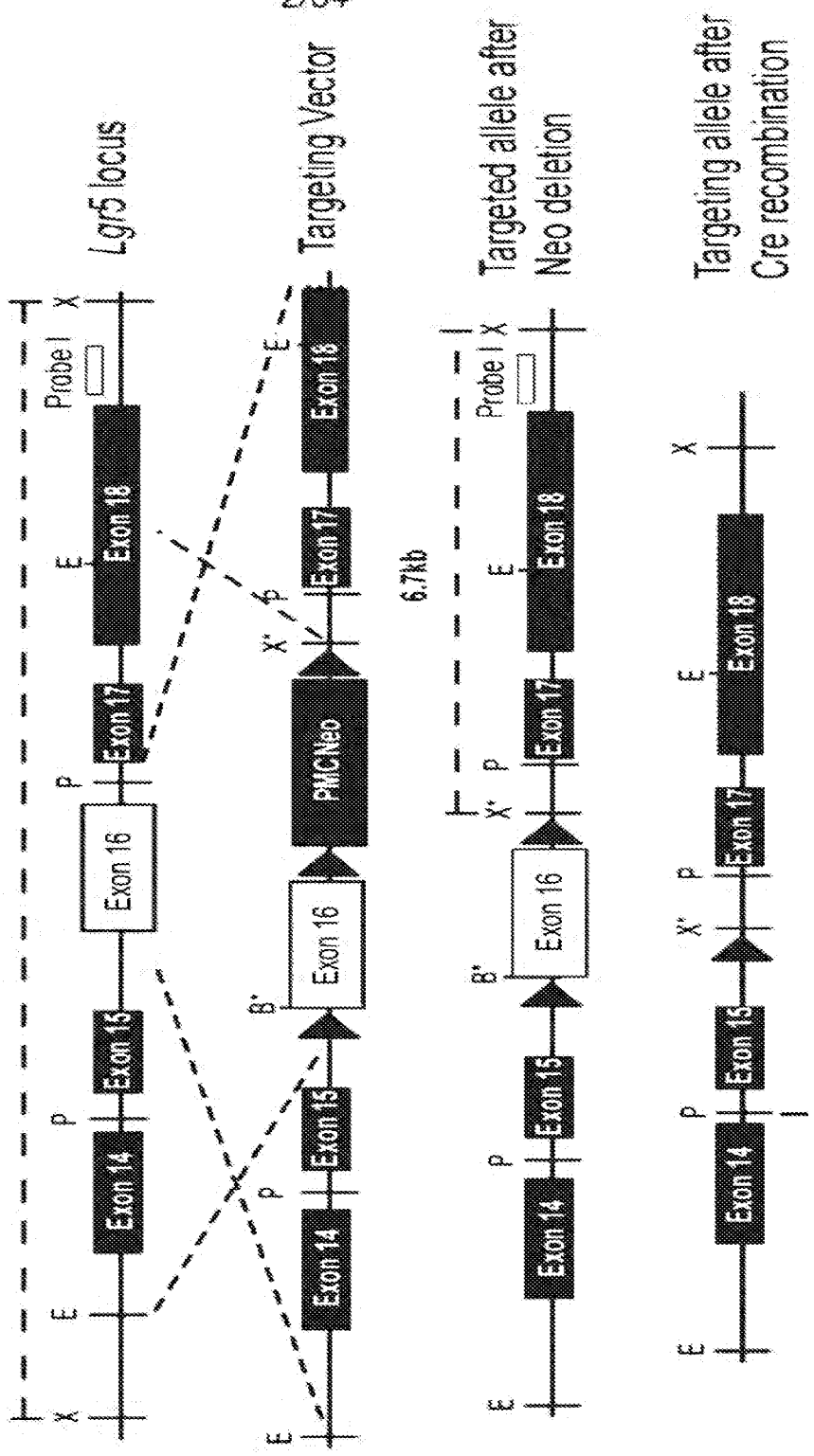

Lgr5-lacZ
Cells expressing Lgr are stained darker
Lgr4-lacZ

FIG. 1(b)


Lgr5 locus
Targeting Vector
Targeted allele after Neo deletion
Targeting allele after Cre recombination
16.7kb
6.7kb
Probe I
Exon 14
Exon 15
Exon 16
Exon 17
Exon 18
PMCNeo
X
E
P
X'
B'

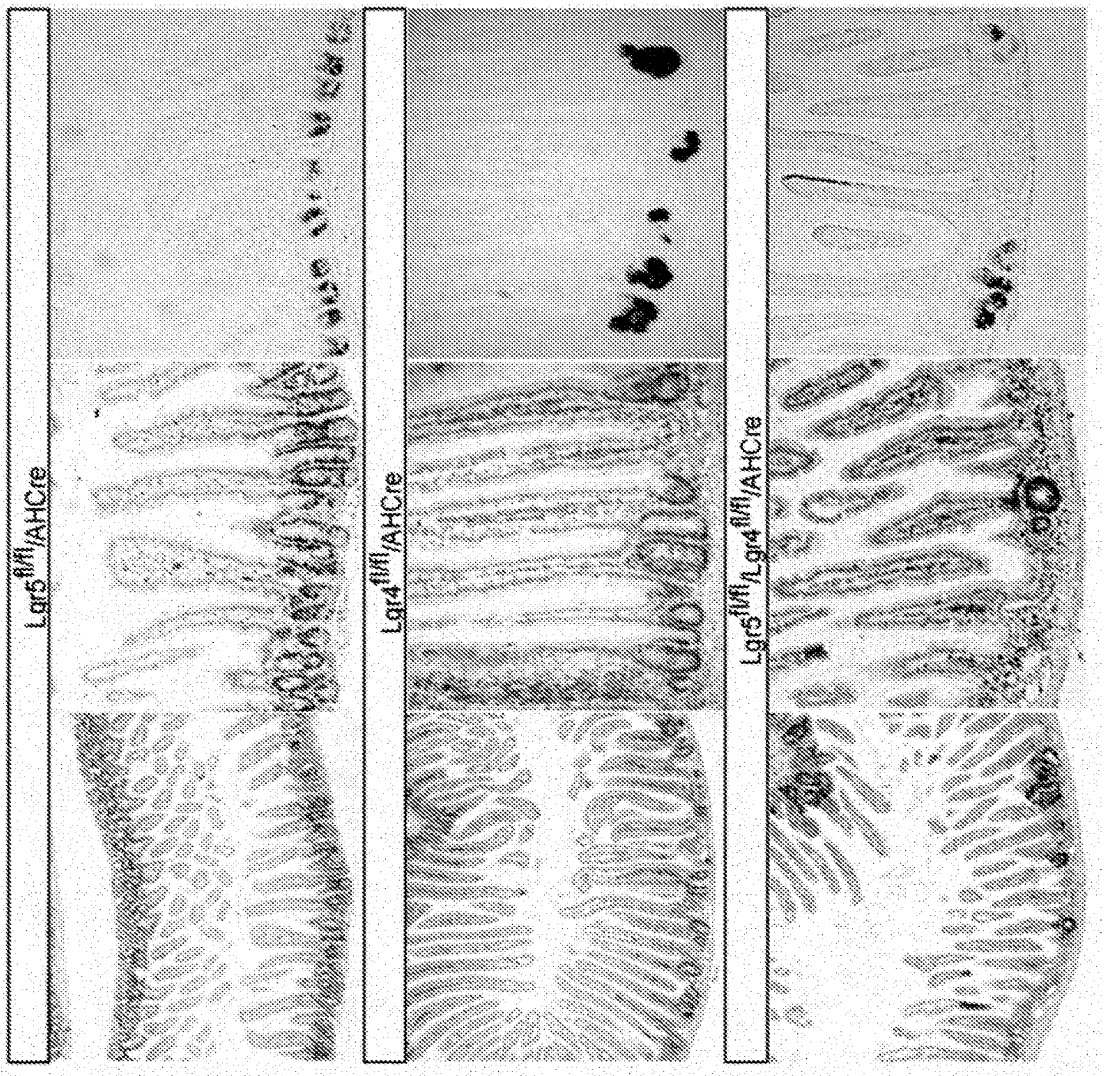


FIG. 1(c)

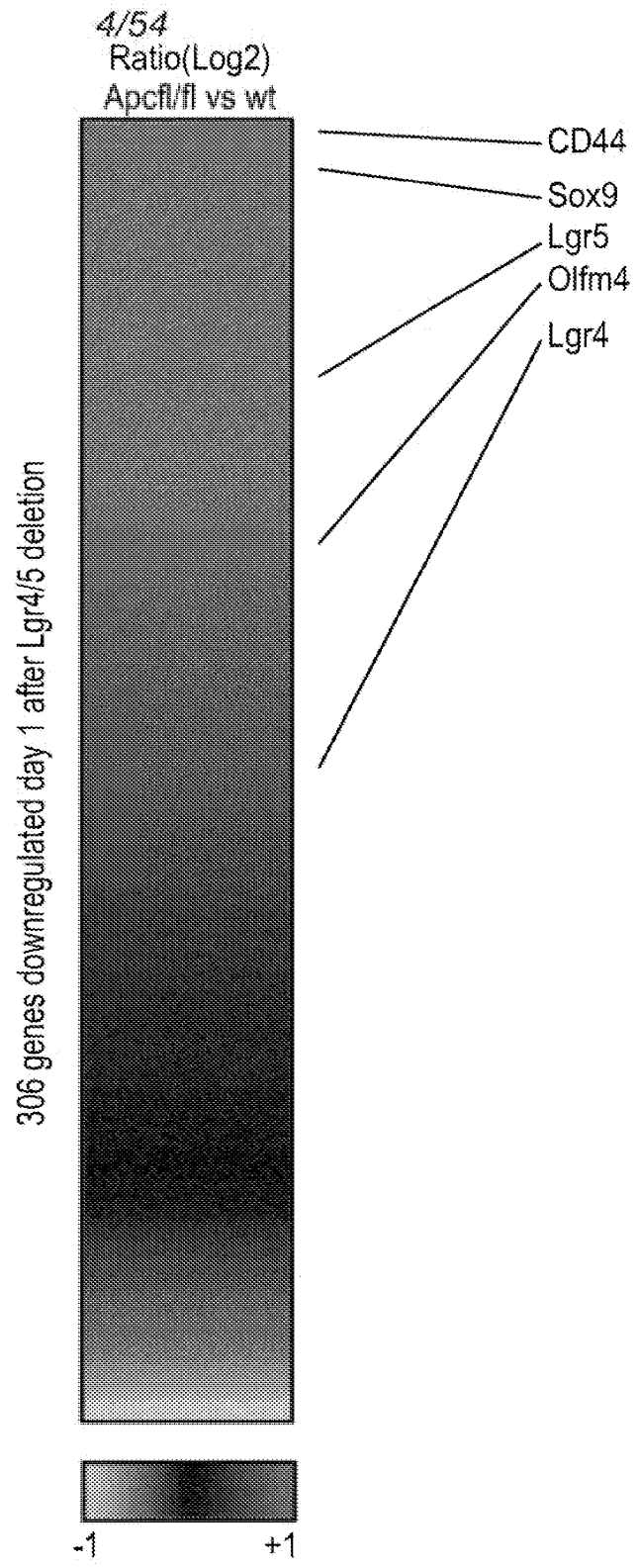

4/54
Ratio(Log2)
Apcfl/fl vs wt
CD44
Sox9
Lgr5
Olfm4
Lgr4
306 genes downregulated day 1 after Lgr4/5 deletion
-1
+1

FIG. 2(A)

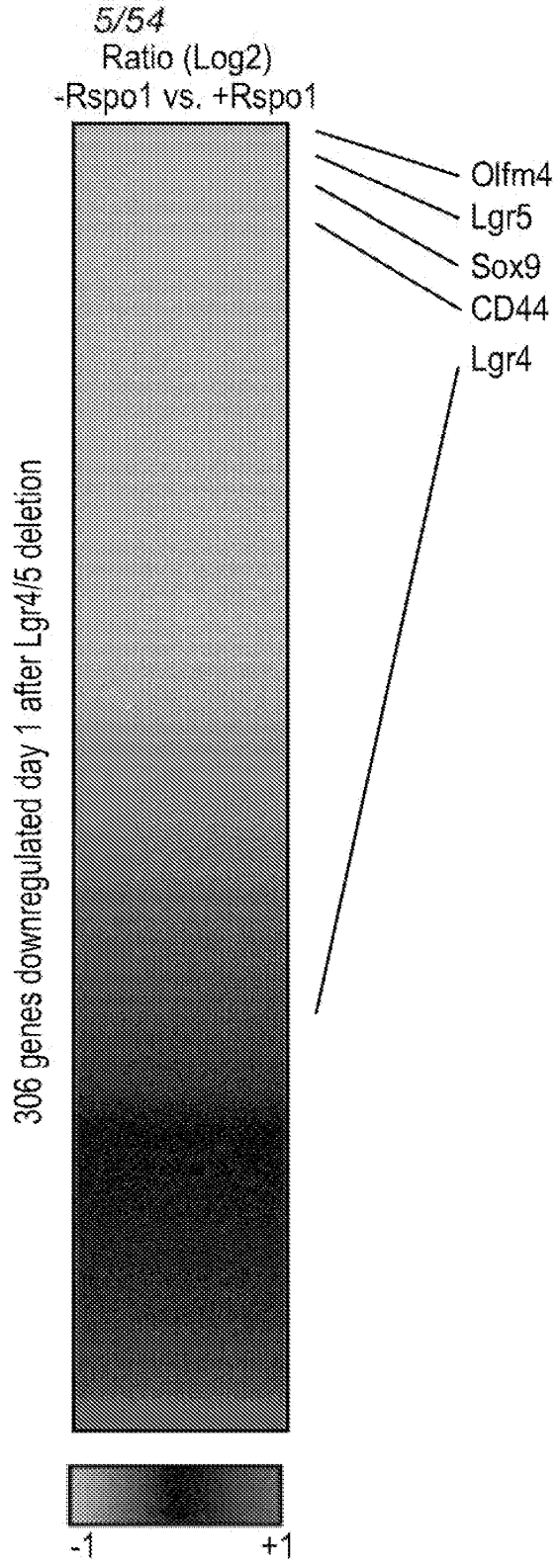

Ratio (Log2)
-Rspo1 vs. +Rspo1
Olfm4
Lgr5
Sox9
CD44
Lgr4
306 genes downregulated day 1 after Lgr4/5 deletion
-1
+1

FIG. 2(B)

FIG. 2(C)

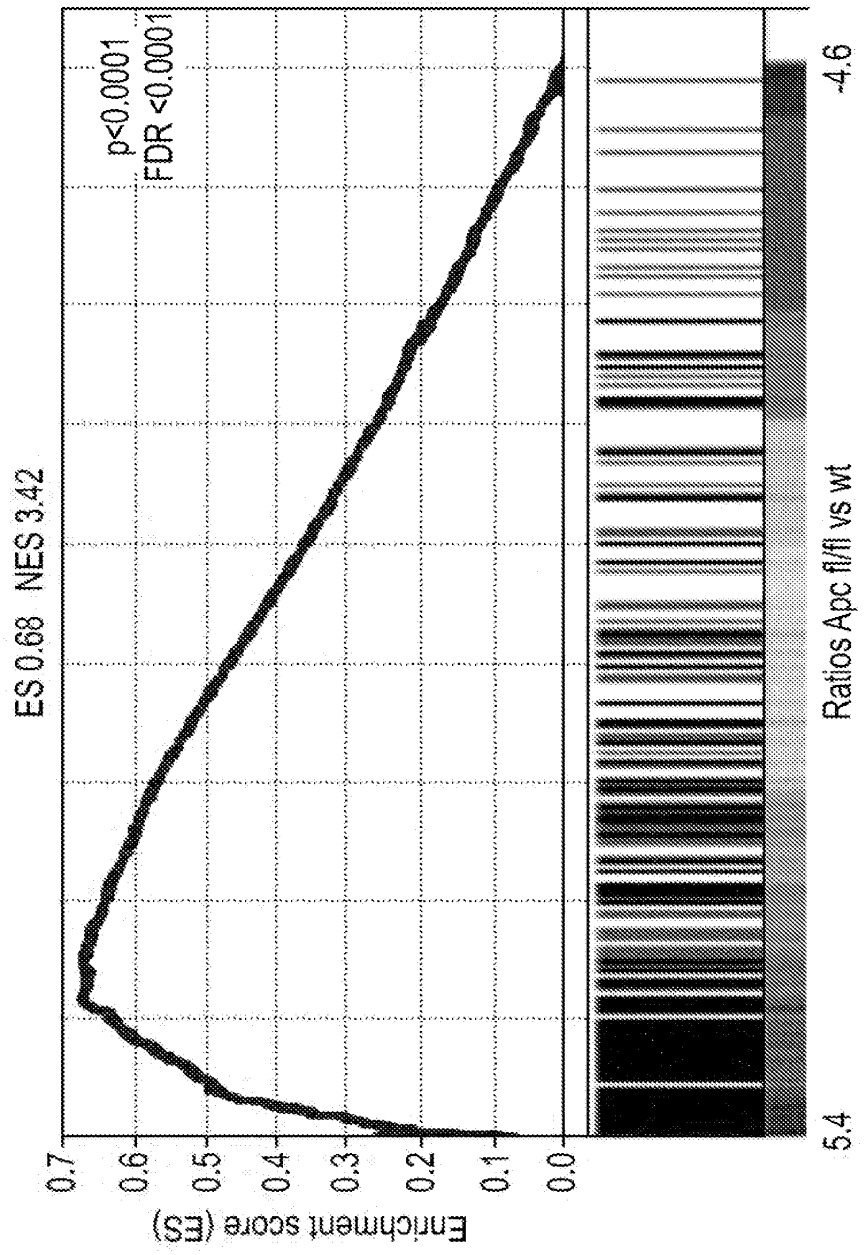
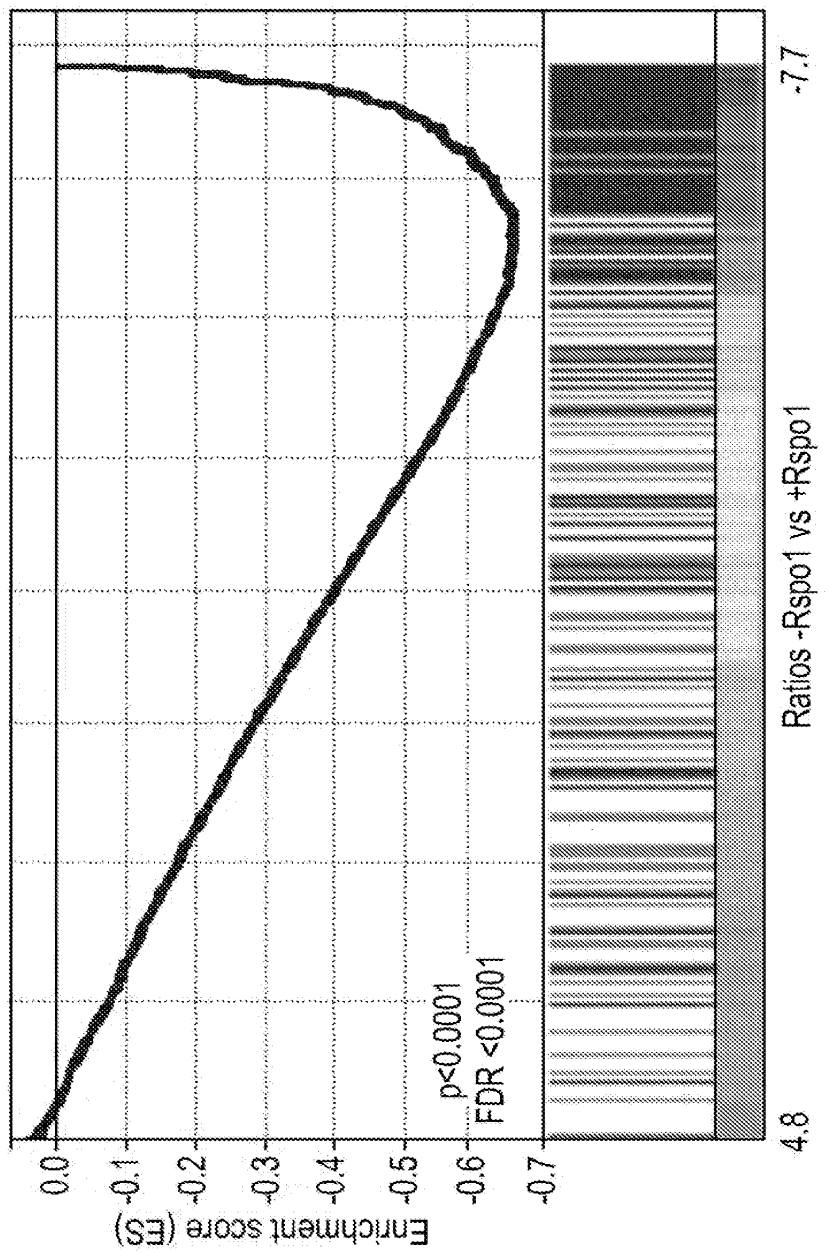

ES 0.68 NES 3.42
p<0.0001
FDR <0.0001
Enrichment score (ES)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5.4
-4.6
Ratios Apc fl/fl vs wt

FIG. 2(D)


ES -0.67 NES -2.89
Enrichment score (ES)
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
p<0.0001
FDR <0.0001
Ratios -Rspo1 vs +Rspo1
4.8
-7.7

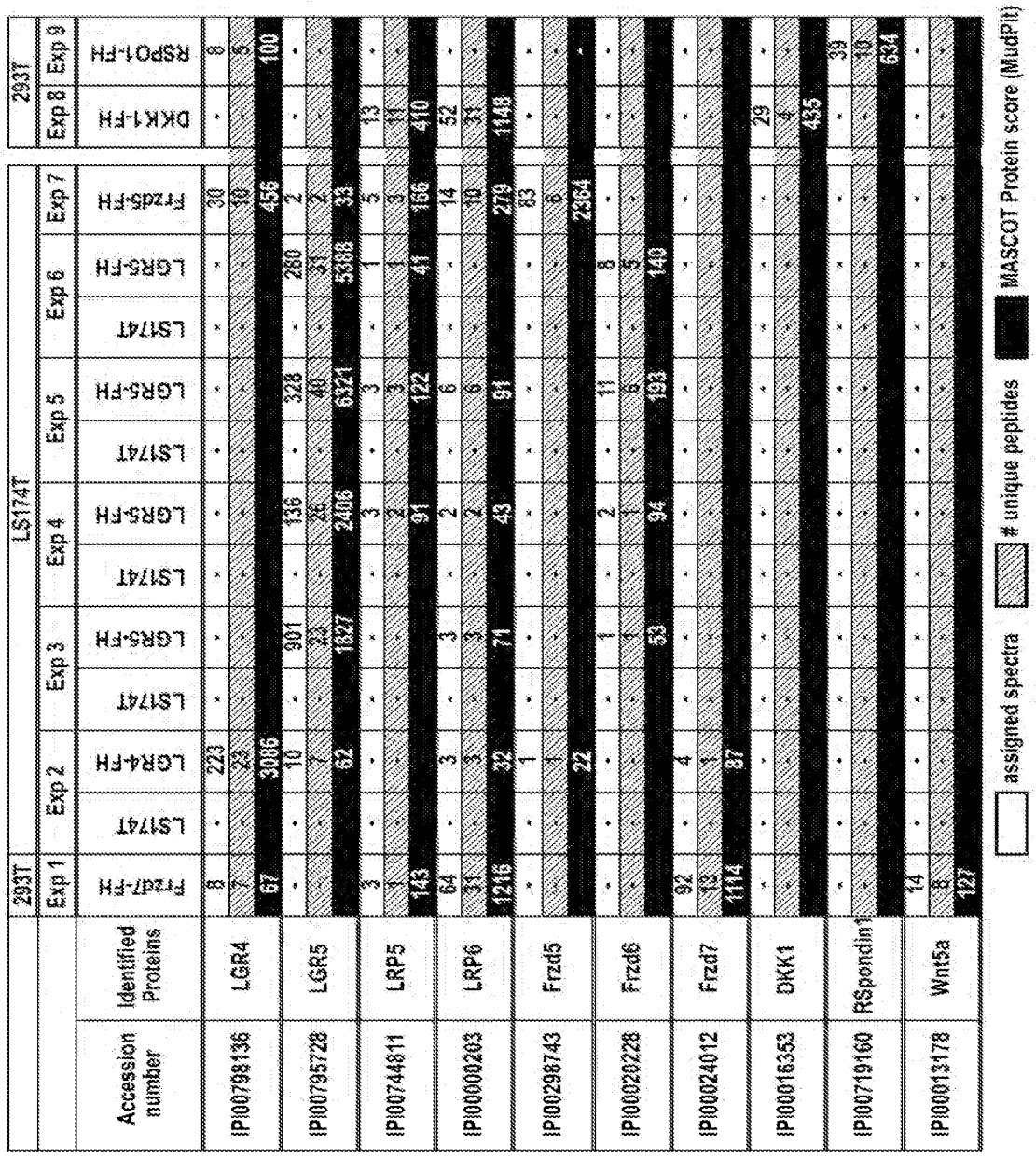

293T
LS174T
293T
Exp 1
Exp 2
Exp 3
Exp 4
Exp 5
Exp 6
Exp 7
Exp 8
Exp 9
Accession number
Identified Proteins
Frzd7-FH
LS174T
LGR4-FH
LS174T
LGR5-FH
LS174T
LGR5-FH
LS174T
LGR5-FH
Frzd5-FH
DKK1-FH
RSPO1-FH
IPI00798136 LGR4
IPI00795728 LGR5
IPI00744811 LRP5
IPI00000203 LRP6
IPI00298743 Frzd5
IPI00020228 Frzd6
IPI00024012 Frzd7
IPI00016353 DKK1
IPI00719160 RSpondin1
IPI00013178 Wnt5a
assigned spectra
unique peptides
MASCOT Protein score (MudPit)

FIG. 3

FIG. 4(A)

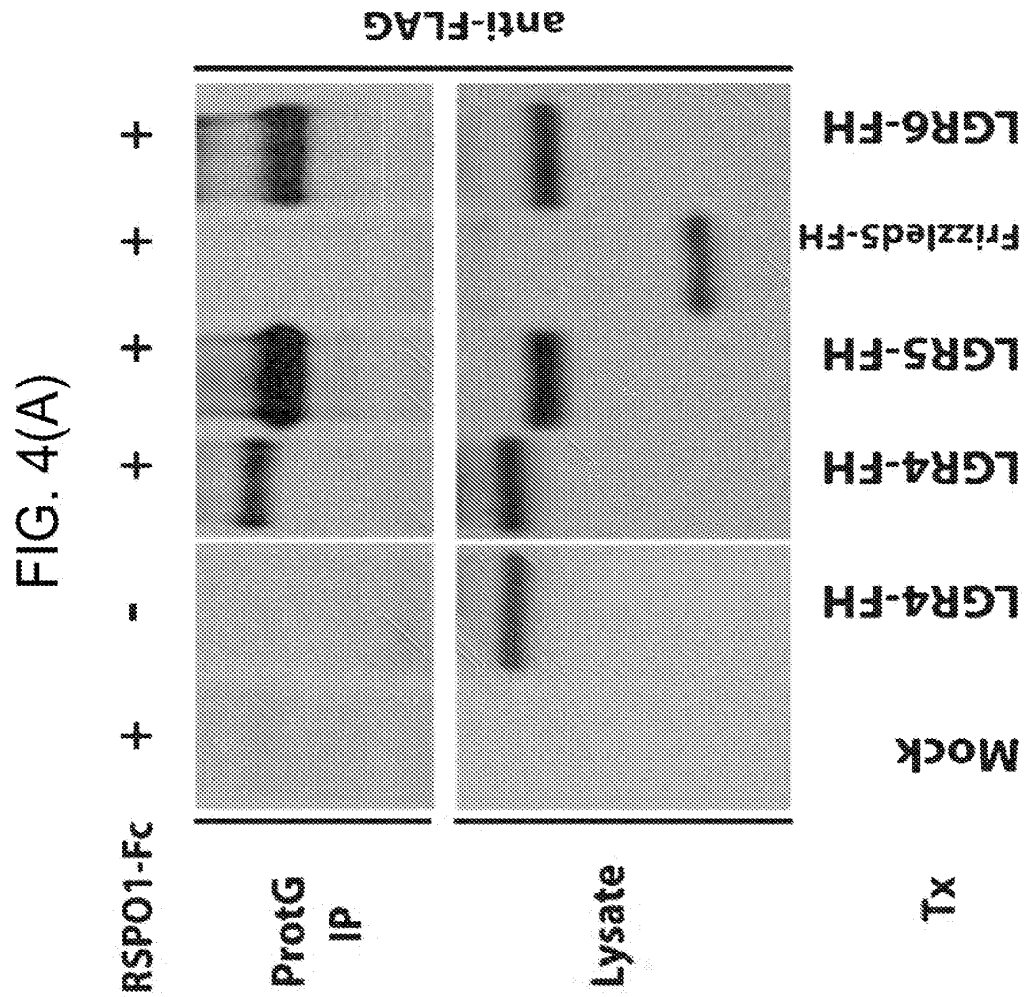
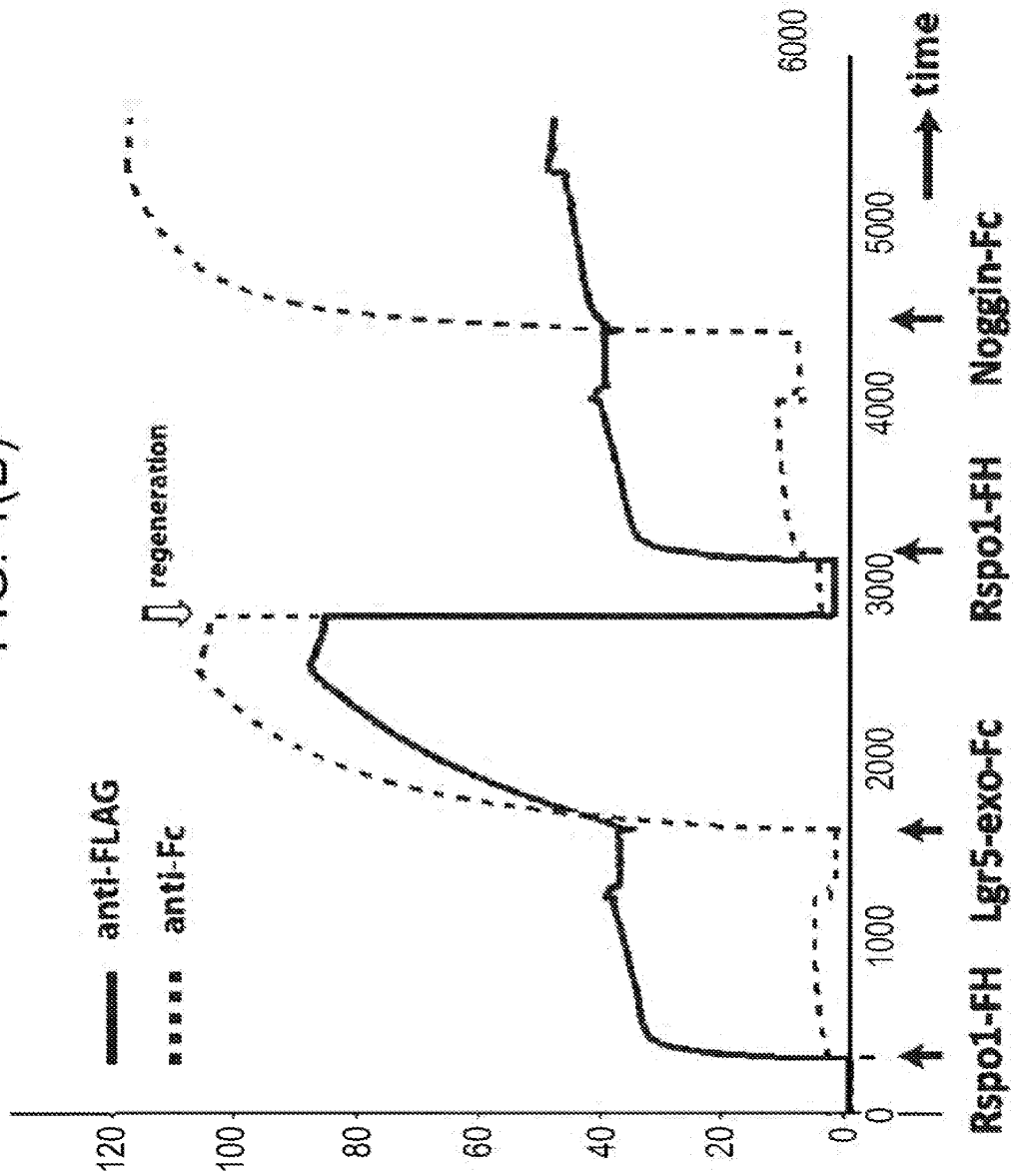

anti-FLAG
LGR6-FH
Frizzled5-FH
LGR5-FH
LGR4-FH
LGR4-FH
Mock
Tx
Lysate
ProtG IP
RSPO1-Fc
+
+
+
+
-
+

FIG. 4(B)


anti-FLAG
anti-Fc
regeneration
120
100
80
60
40
20
0
0
1000
2000
3000
4000
5000
6000
time
Rspo1-FH
Lgr5-exo-Fc
Rspo1-FH
Noggin-Fc

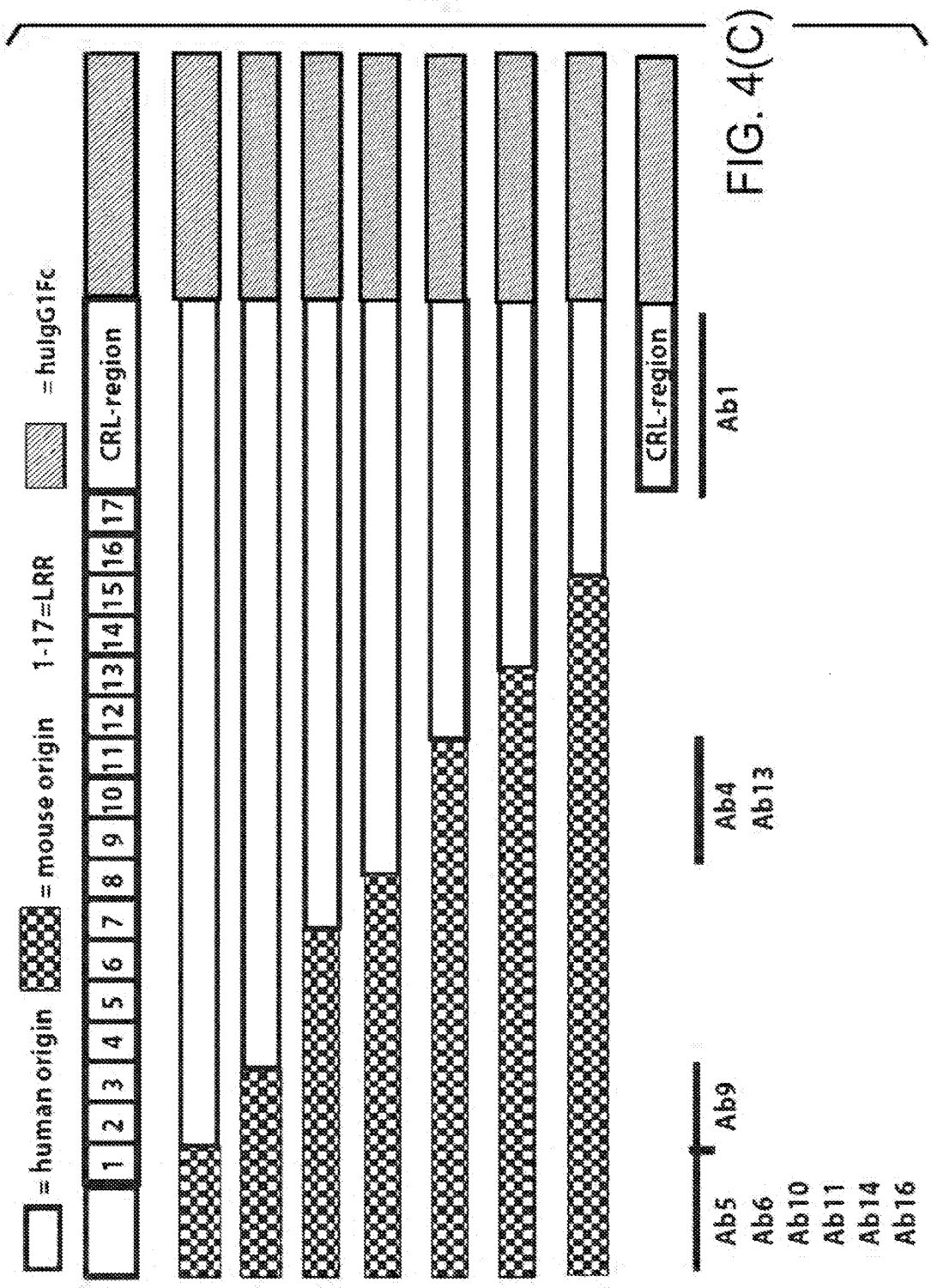

= human origin
= mouse origin
1-17=LRR
= huIgG1Fc
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
CRL-region
Ab5
Ab9
Ab6
Ab10
Ab11
Ab14
Ab16
Ab4
Ab13
CRL-region
Ab1

FIG. 4(C)

FIG. 4(D)

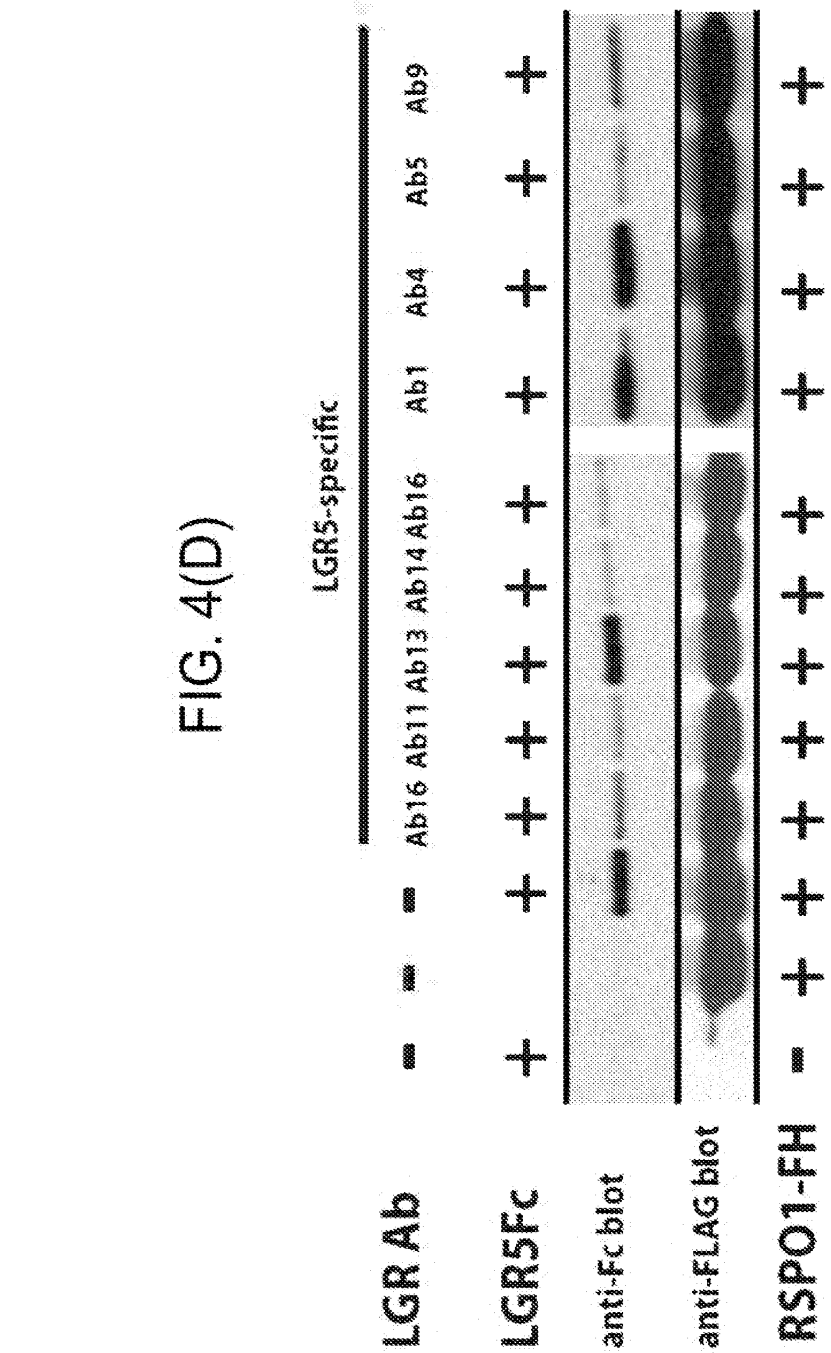
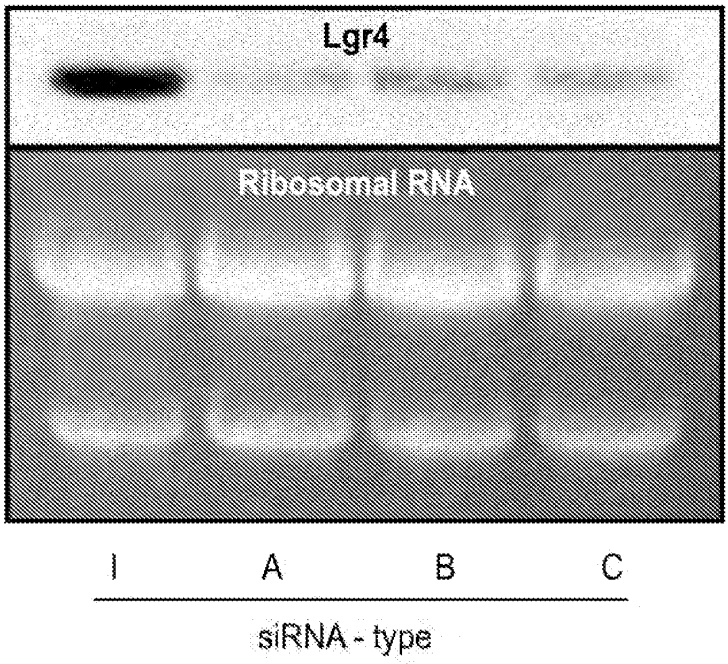

LGR5-specific
LGR Ab
- - - Ab16 Ab11 Ab13 Ab14 Ab16 Ab1 Ab4 Ab5 Ab9
LGR5Fc
+ + + + + + + + + +
anti-Fc blot
anti-FLAG blot
RSPO1-FH
- + + + + + + + + +

FIG. 5(A)



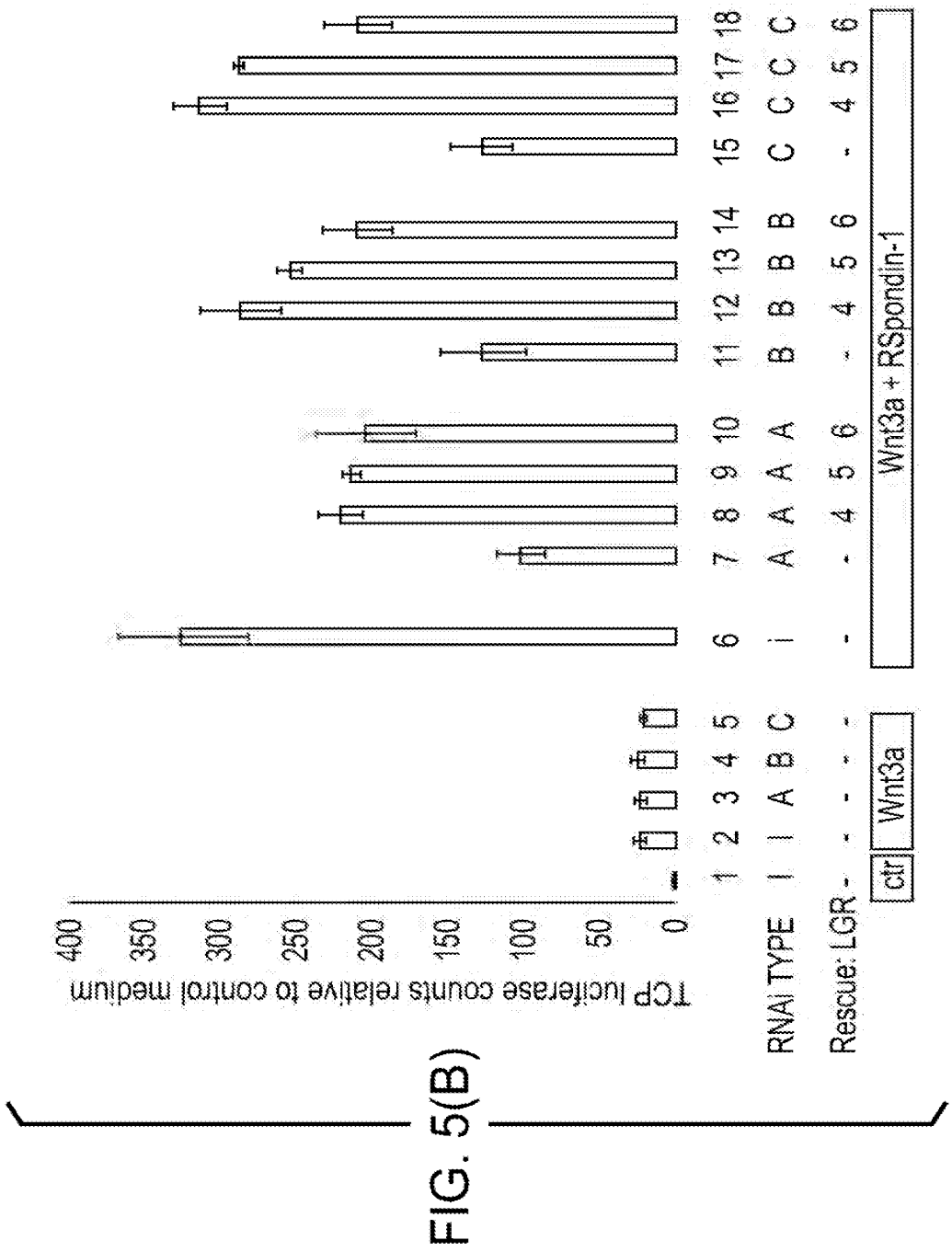

TCP luciferase counts relative to control medium
400
350
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
RNAi TYPE I I A B C i A A A A B B B B C C C C
Rescue: LGR - - - - - - - 4 5 6 - 4 5 6 - 4 5 6
ctr
Wnt3a
Wnt3a + RSpondin-1

FIG. 5(B)

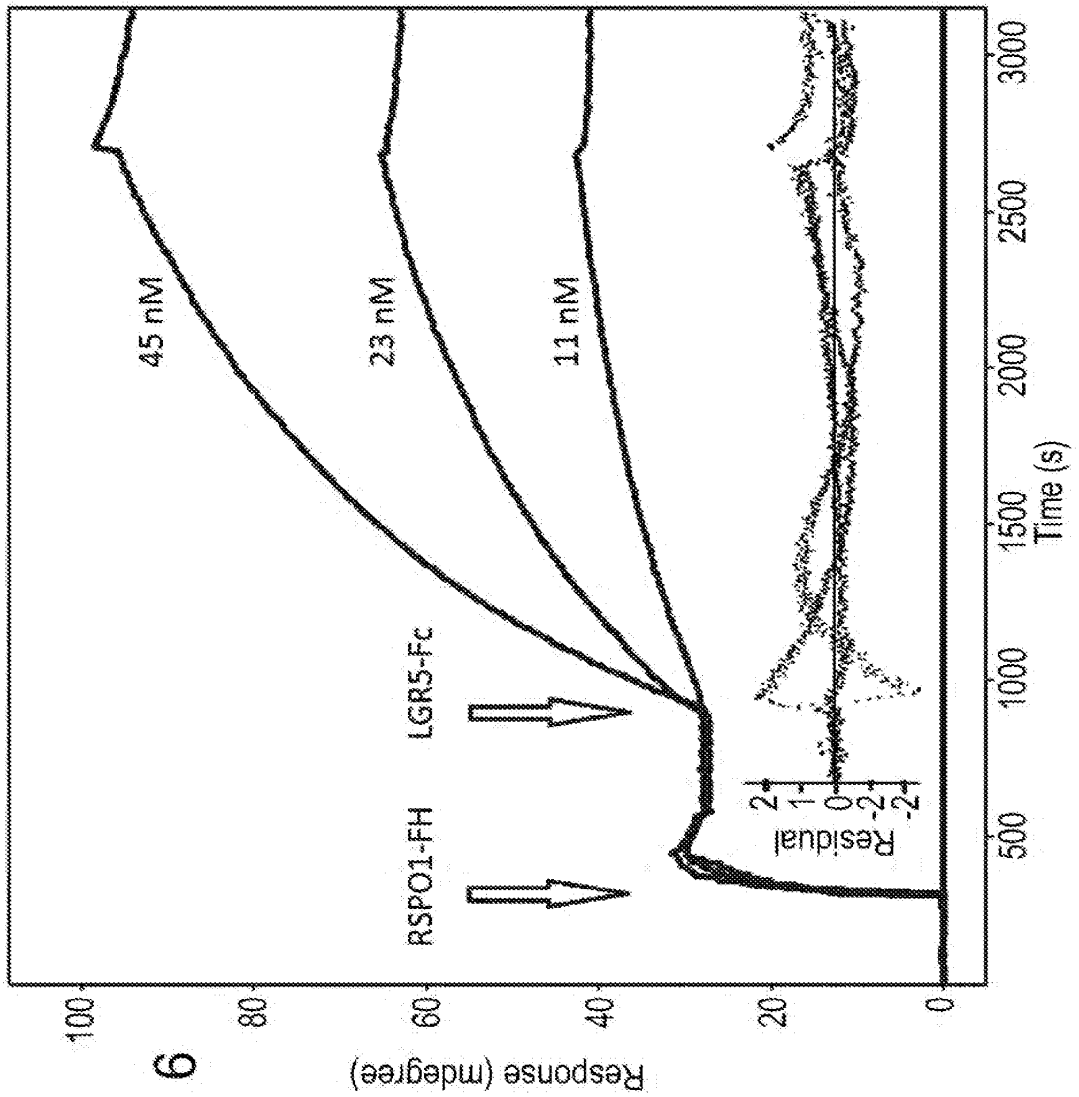

45 nM
23 nM
11 nM
RSPO1-FH
LGR5-Fc
Response (mdegree)
Time (s)
Residual
0
20
40
60
80
100
500
1000
1500
2000
2500
3000
2
1
0
-2
-2

FIG. 6

FIG. 7

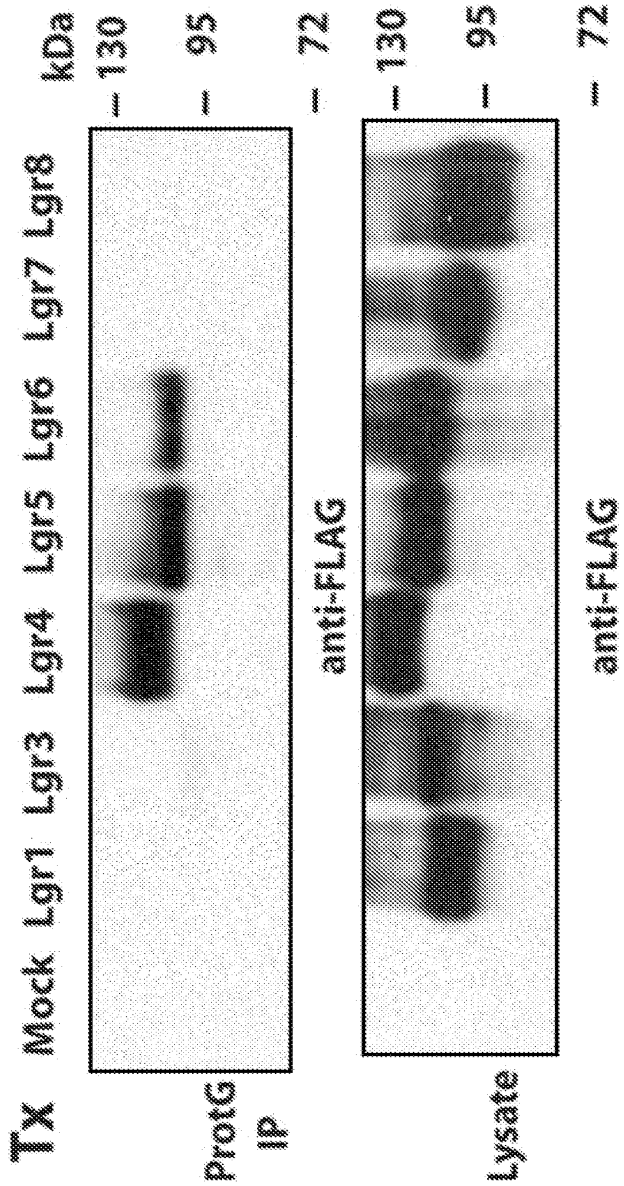

Tx
Mock
Lgr1
Lgr3
Lgr4
Lgr5
Lgr6
Lgr7
Lgr8
kDa
130
95
72
ProtG IP
anti-FLAG
Lysate
anti-FLAG

FIG. 8A

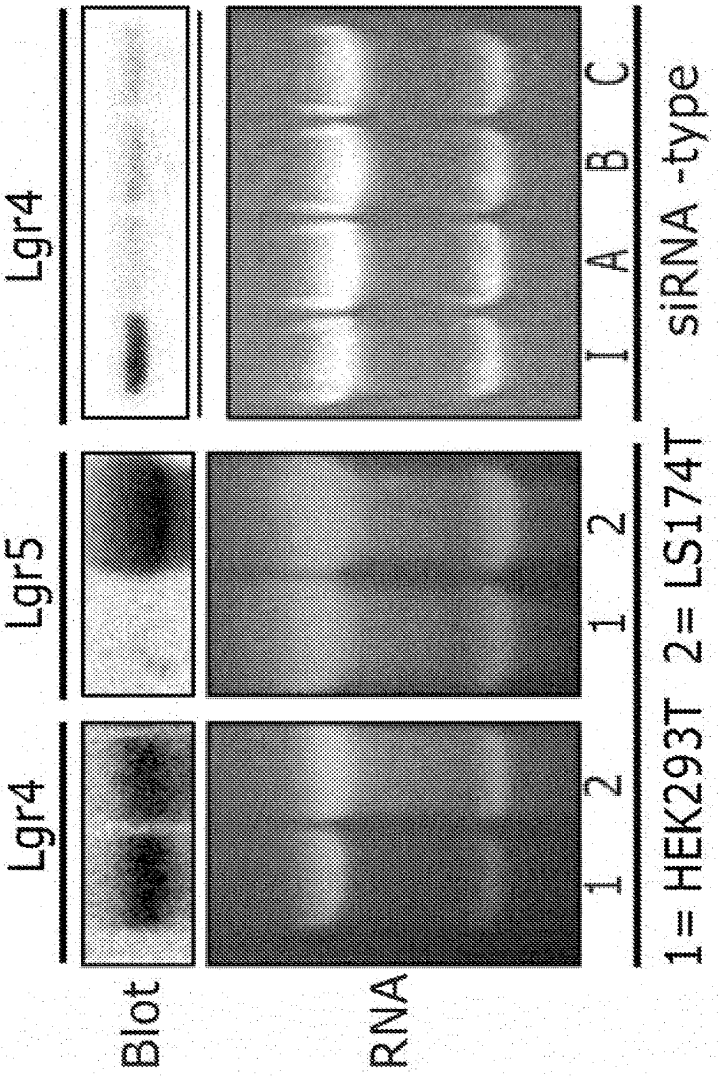

Lgr4
Lgr5
Lgr4
Blot
RNA
1 2
1 2
I A B C
1= HEK293T 2= LS174T
siRNA -type

FIG. 8B

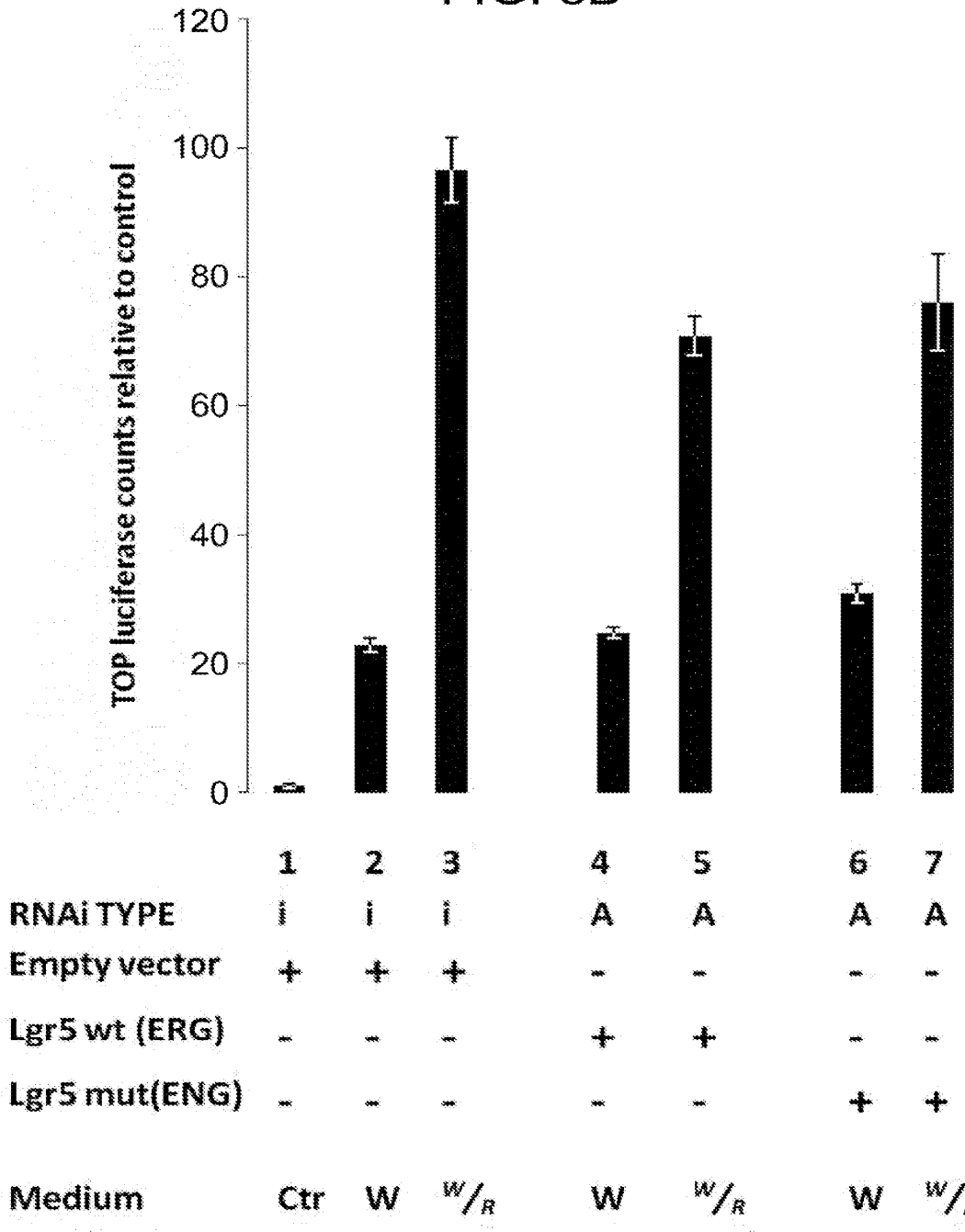

TOP luciferase counts relative to control
120
100
80
60
40
20
0
1 2 3 4 5 6 7
RNAi TYPE i i i A A A A
Empty vector + + + - - - -
Lgr5 wt (ERG) - - - + + - -
Lgr5 mut(ENG) - - - - - + +
Medium Ctr W W/R W W/R W W/R

FIG. 9A

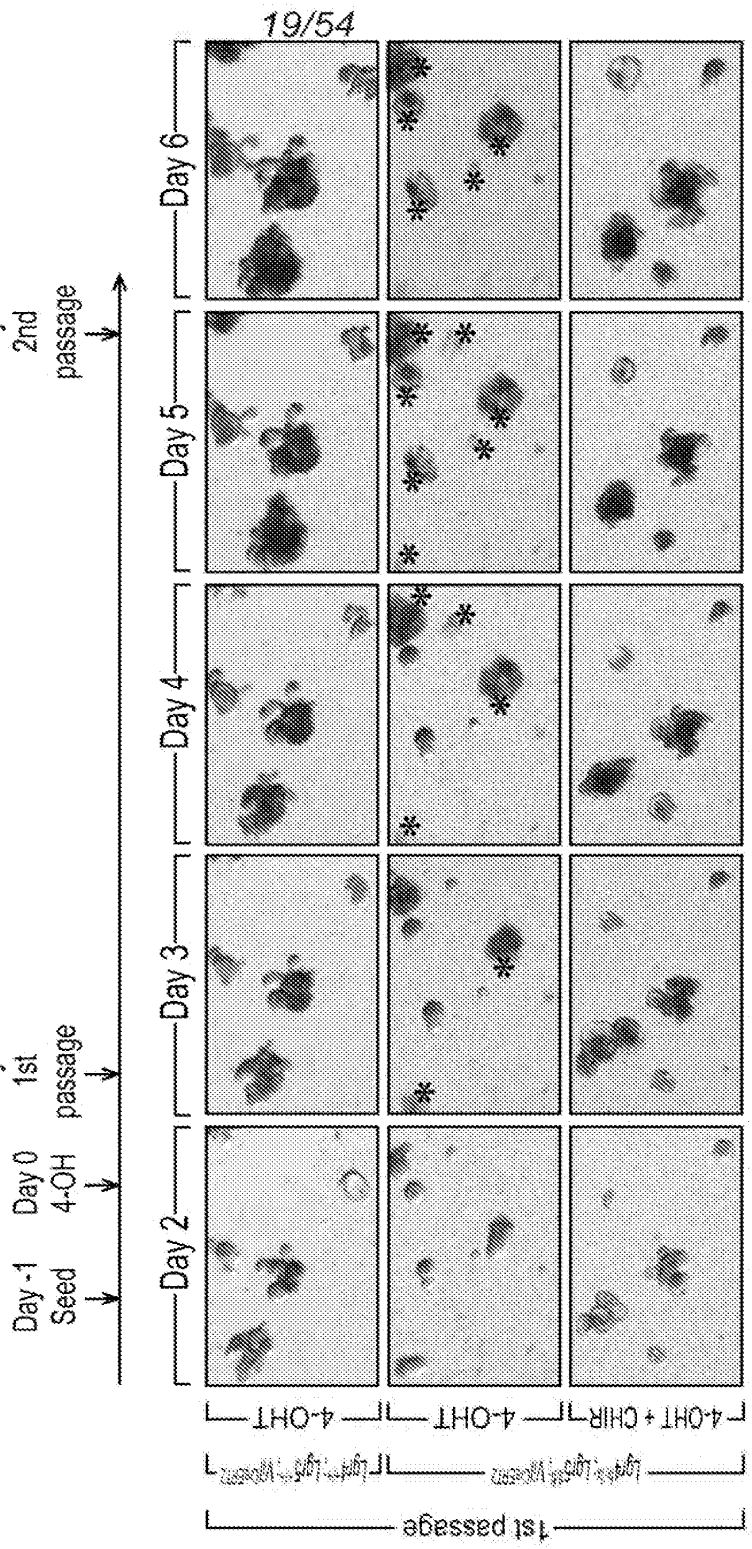

Day -1
Seed
Day 0
4-OH
Day 1
1st
passage
Day 7
2nd
passage
Day 2
Day 3
Day 4
Day 5
Day 6
4-OHT
4-OHT
4-OHT + CHIR
1st passage

FIG. 9B

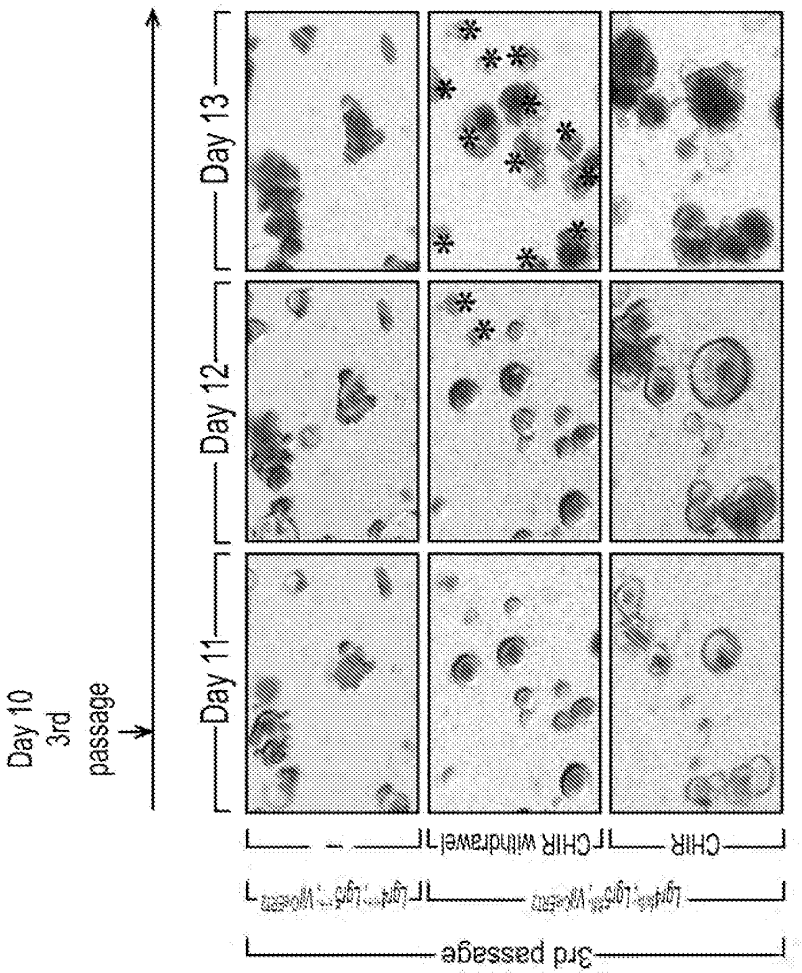

Day 10
3rd
passage
Day 11
Day 12
Day 13
-
CHIR withdrawel
CHIR
3rd passage

FIG. 9C

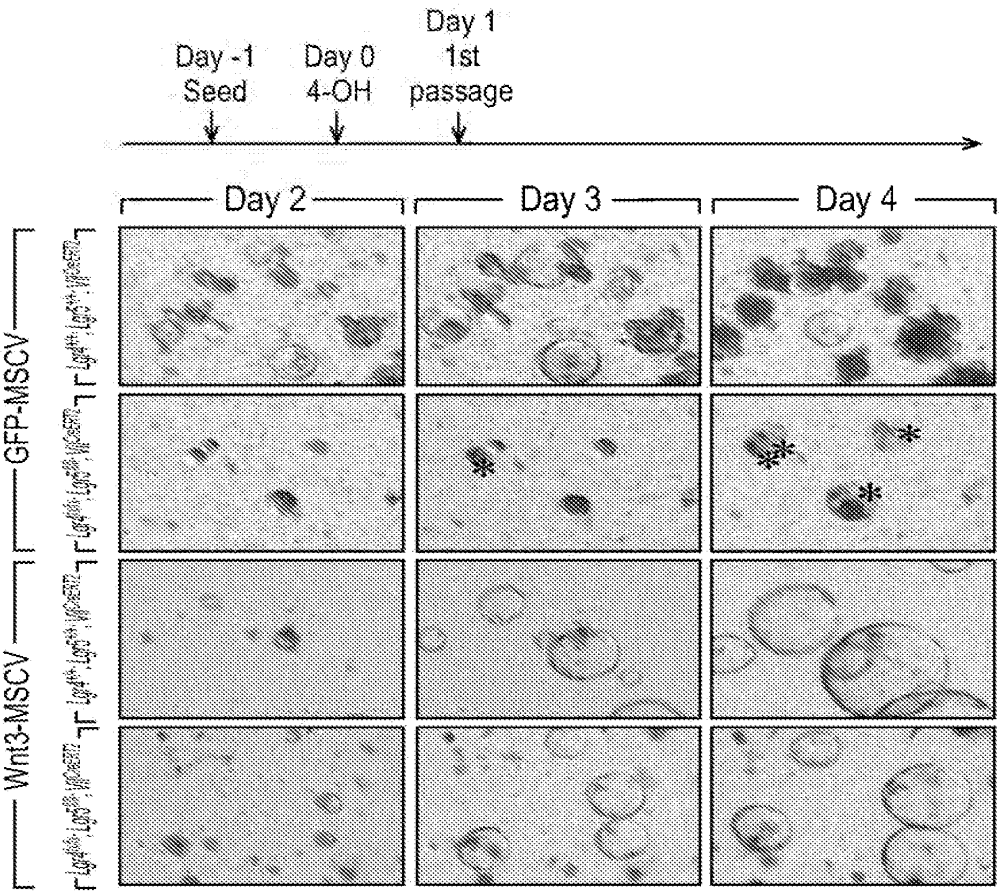

Day -1
Seed
Day 0
4-OH
Day 1
1st
passage
Day 2
Day 3
Day 4
GFP-MSCV
Wnt3-MSCV

FIG. 10

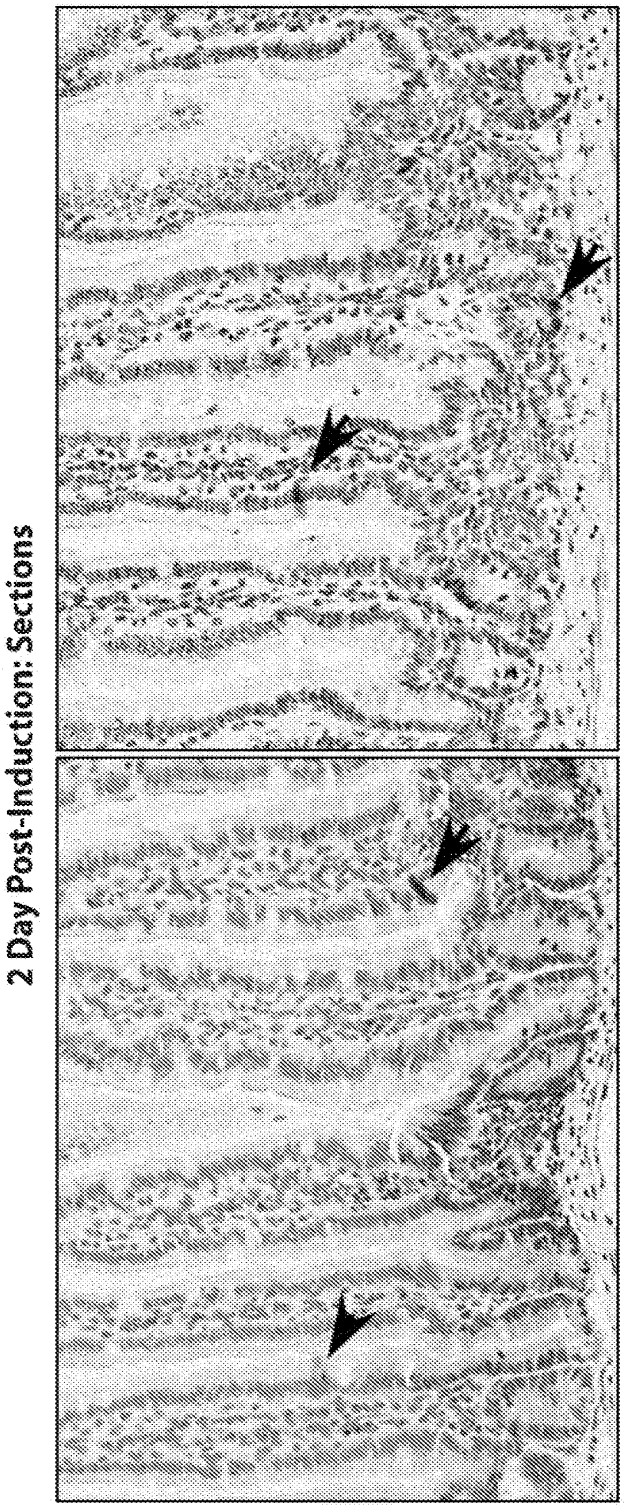


FIG. 10(contd.)

395 Day Post-Induction: Whole-mount

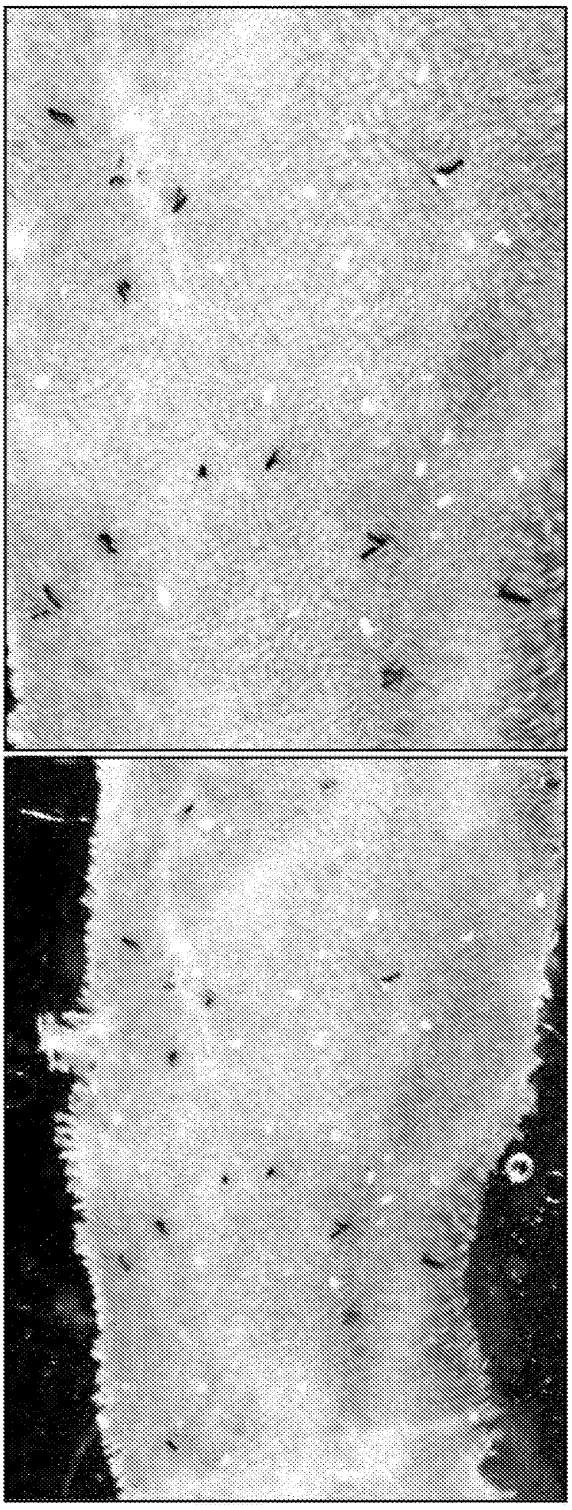


FIG. 10(contd.)

395 Day Post-Induction: Sections

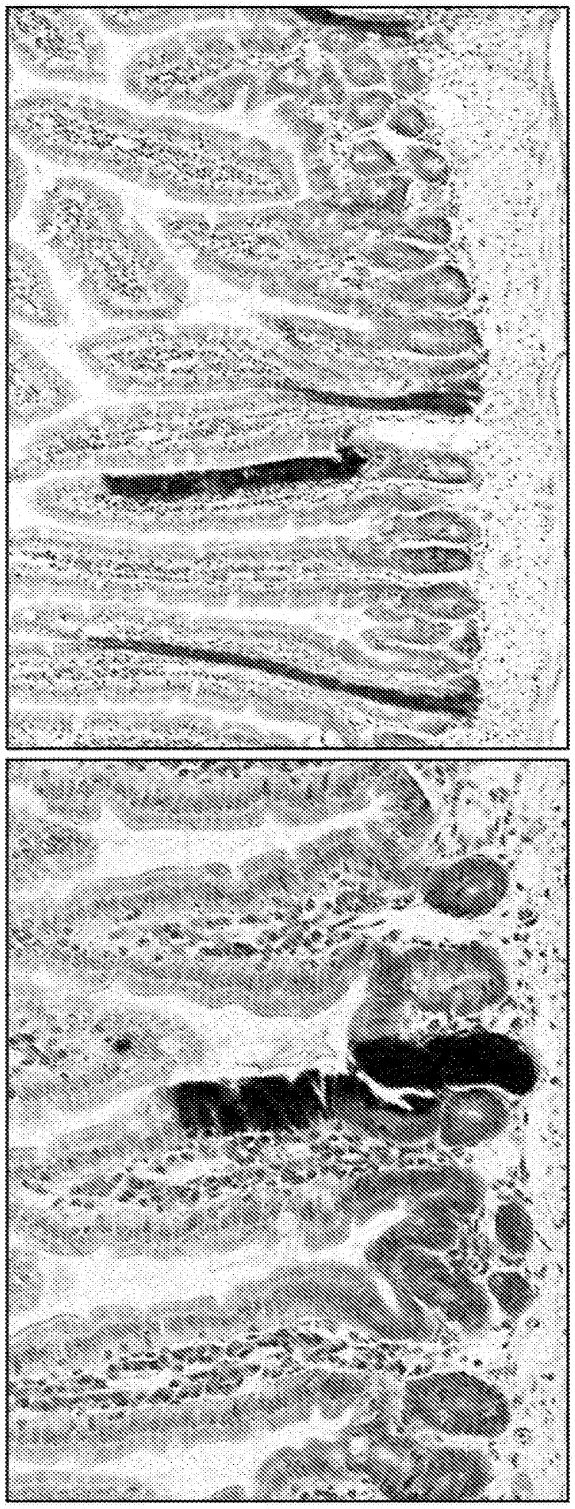


FIG. 11A

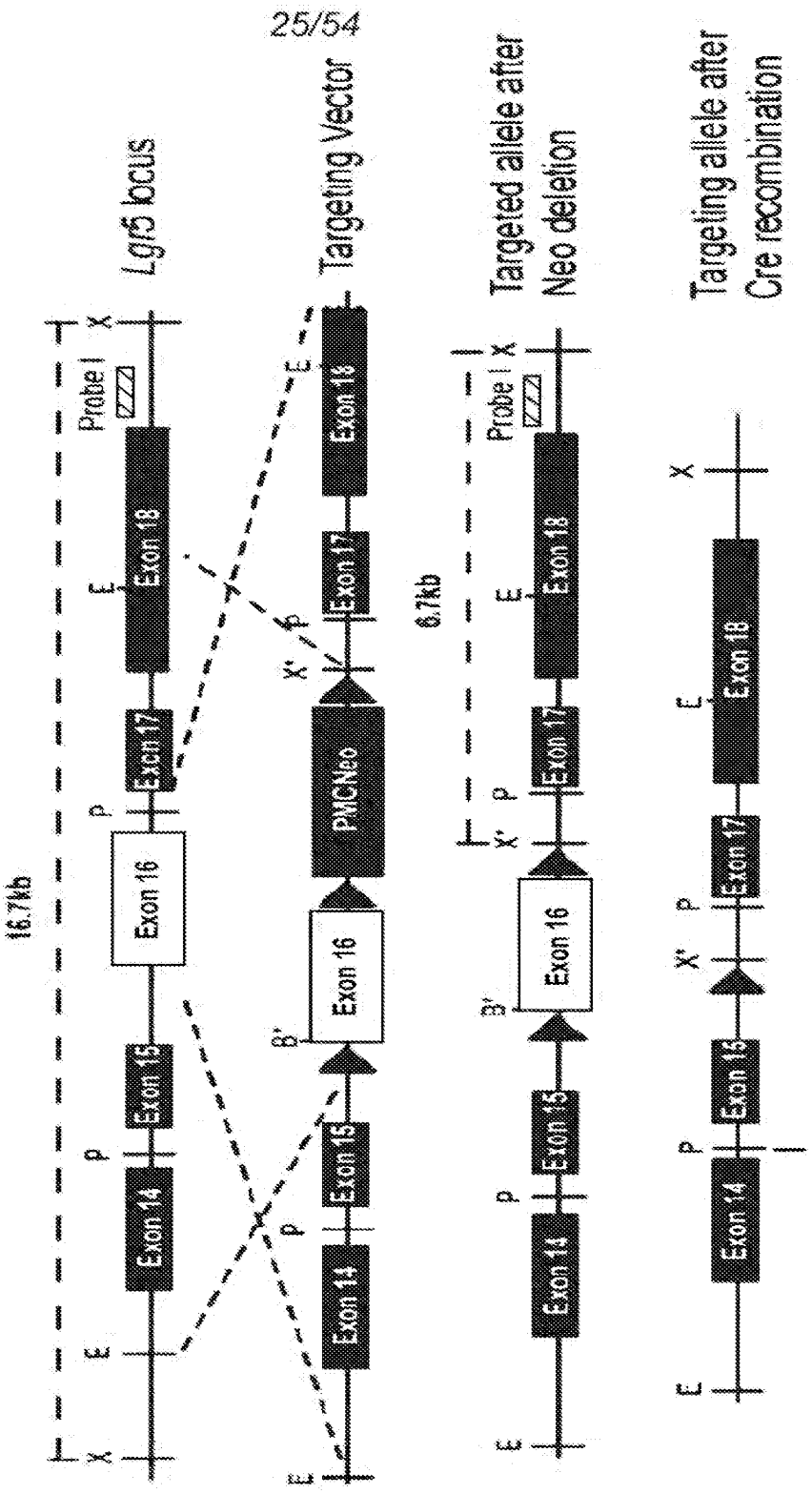
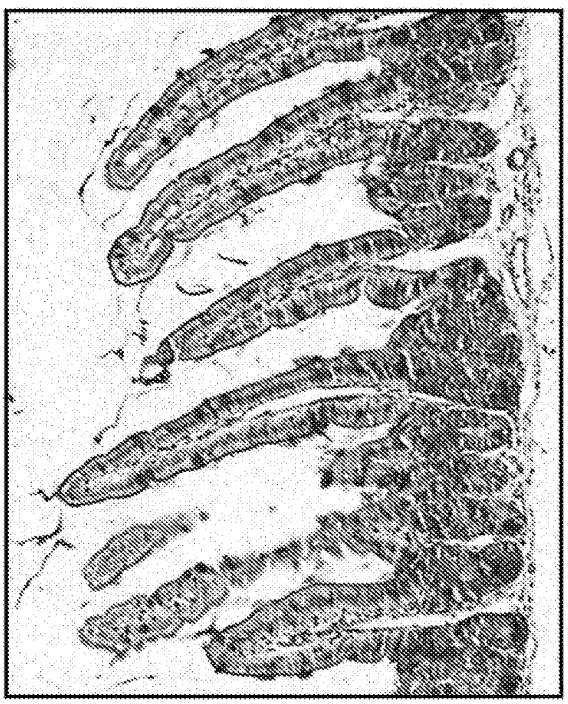

Lgr5 locus
Targeting Vector
Targeted allele after Neo deletion
Targeting allele after Cre recombination
16.7kb
6.7kb
Probe I
Exon 18
Exon 17
Exon 16
Exon 15
Exon 14
PMCNeo
X
E
P
X'
B'

FIG. 11B

PAS staining

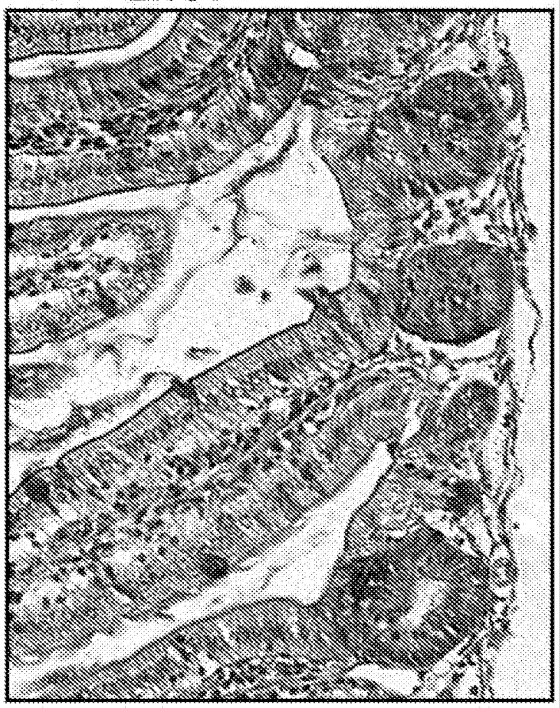




Lgr5fl/fl/AHCre

FIG. 11B (contd.)

PAS staining

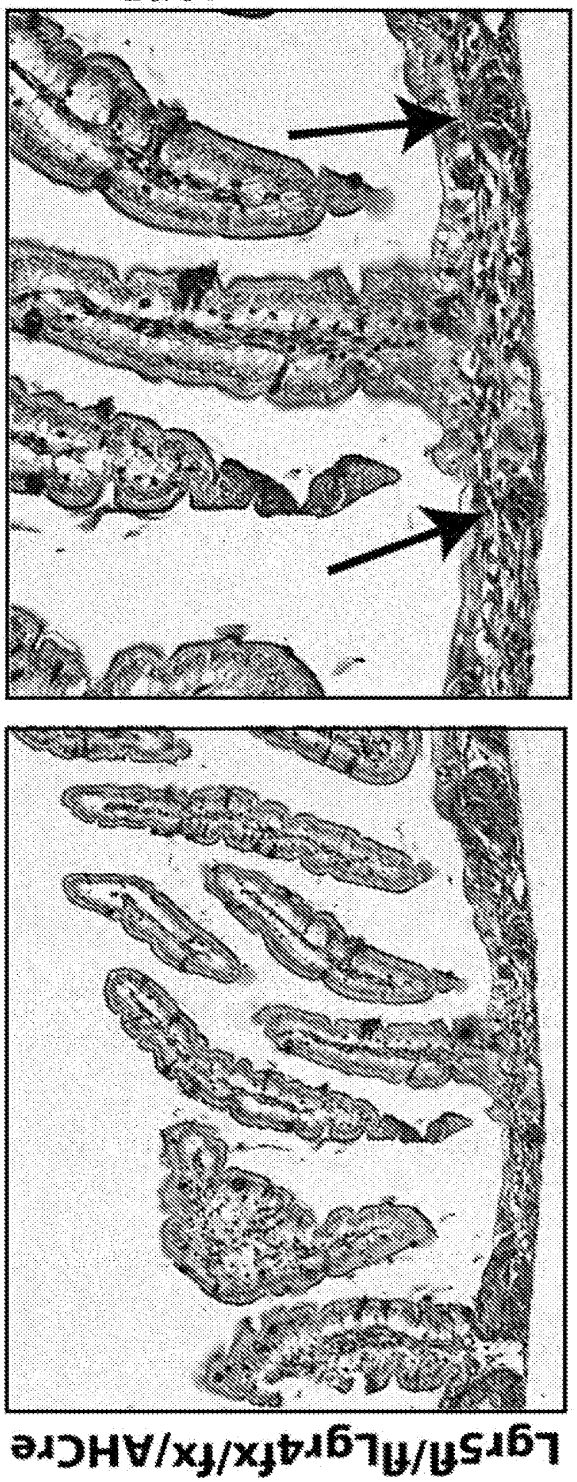




Lgr4fx/fx/AHCre

FIG. 11B (contd.)

PAS staining



Lgr5fl/flLgr4fx/fx/ΔHCre

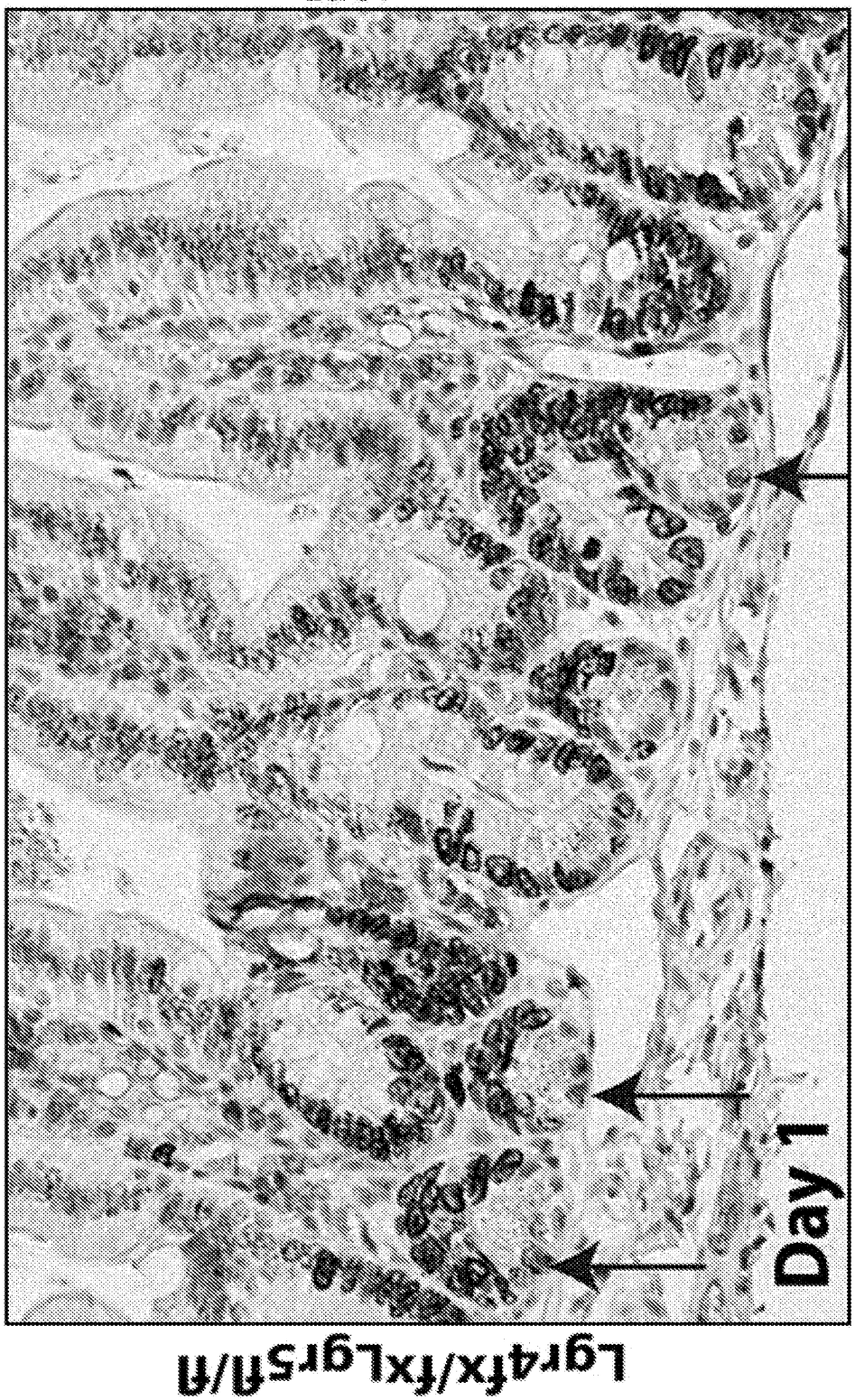


FIG. 12

FIG. 12(contd.)

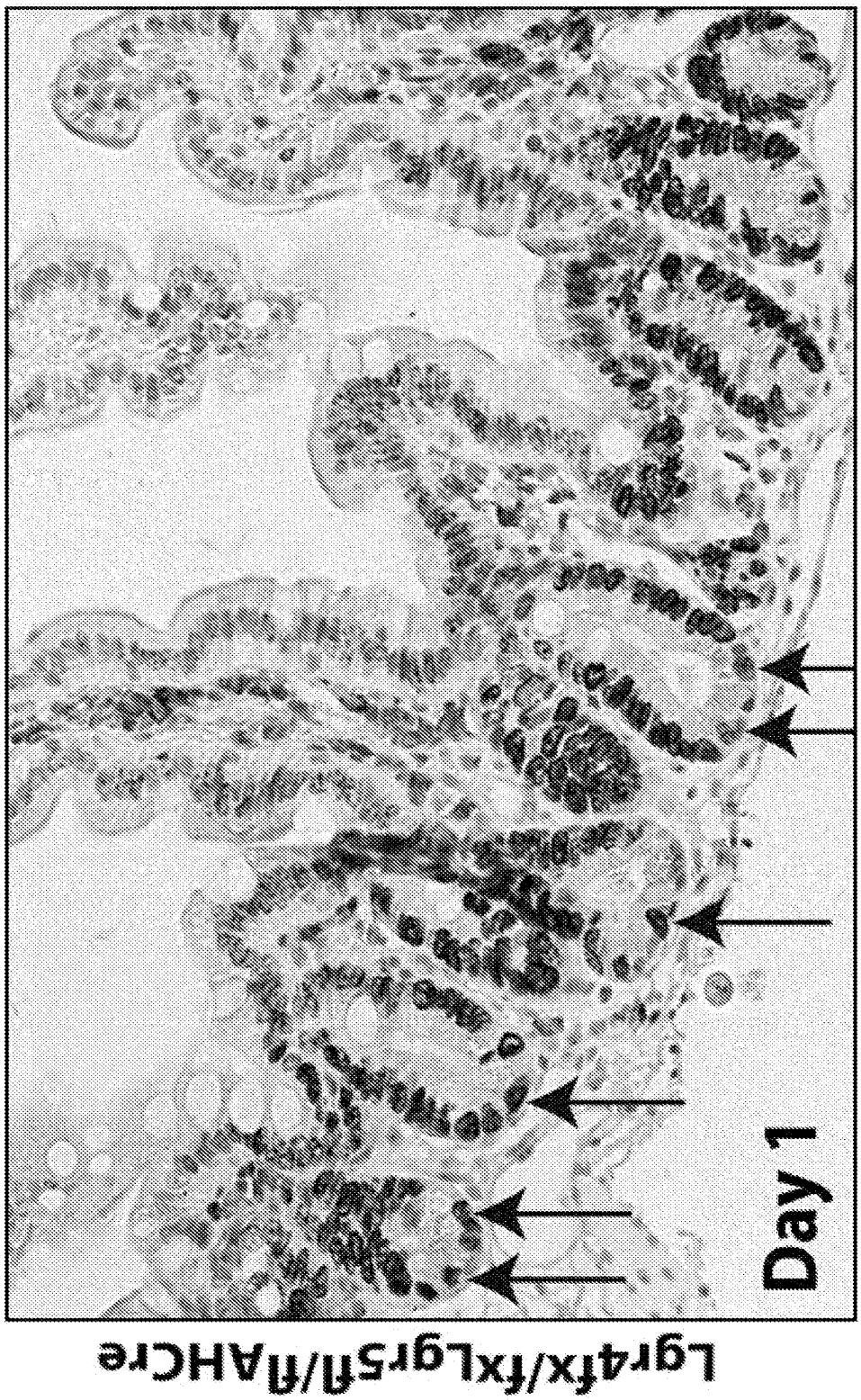
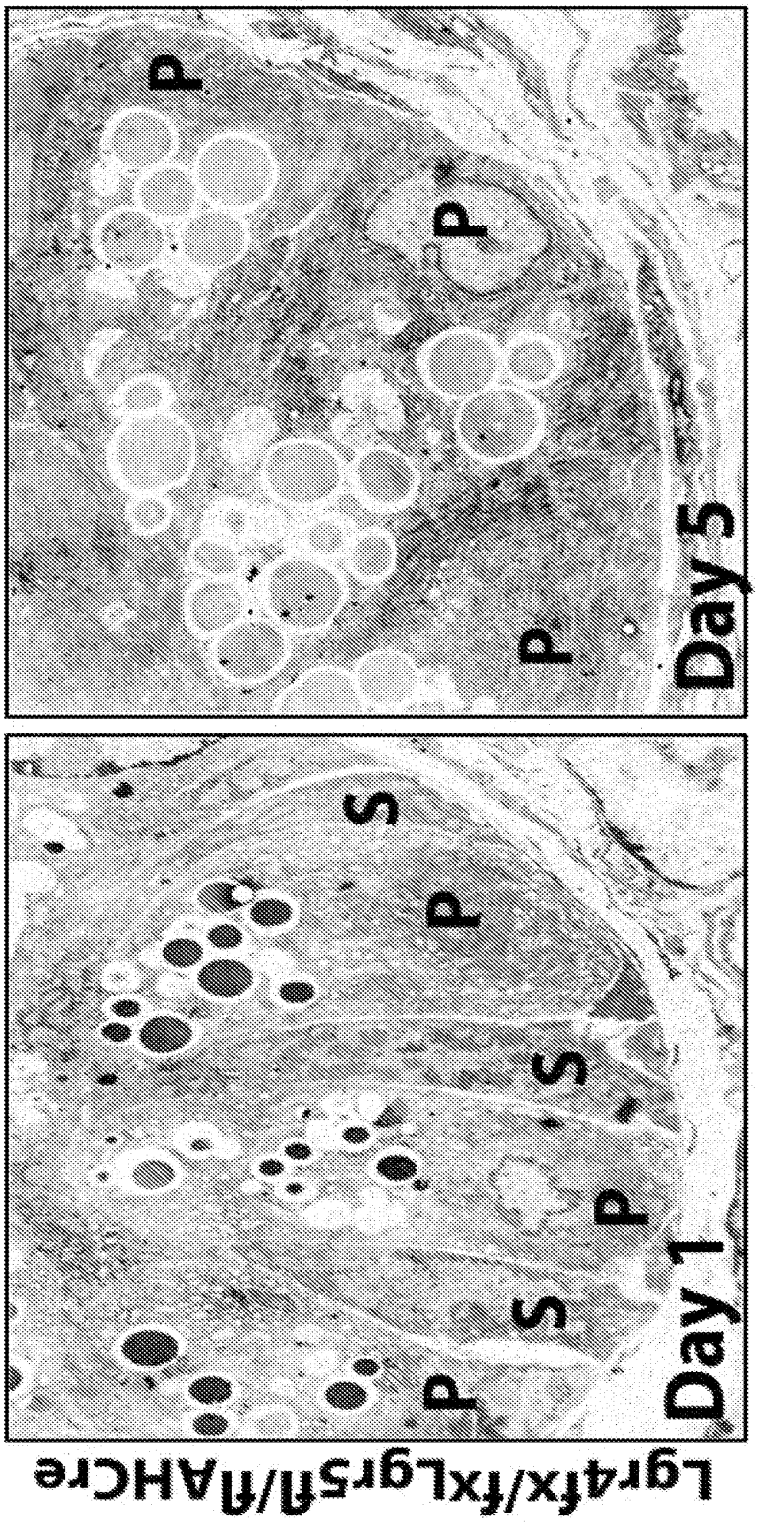


FIG. 12(contd.)



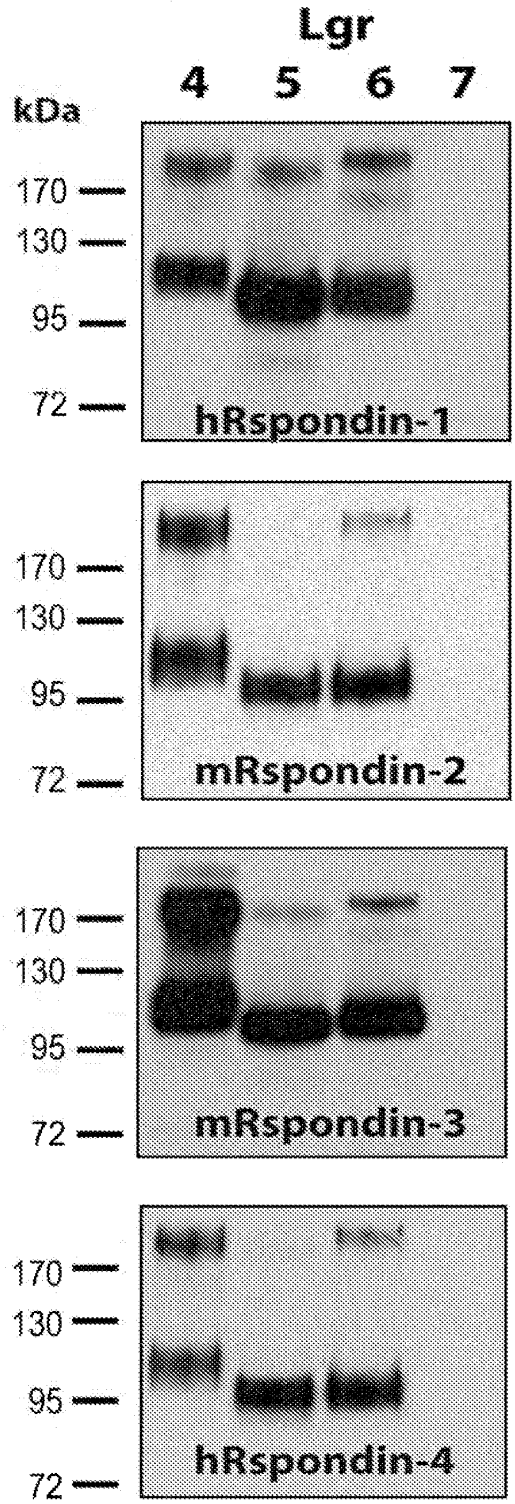

Lgr
4
5
6
7
kDa
170
130
95
72
hRspondin-1
170
130
95
72
mRspondin-2
170
130
95
72
mRspondin-3
170
130
95
72
hRspondin-4

FIG. 13

FIG. 14

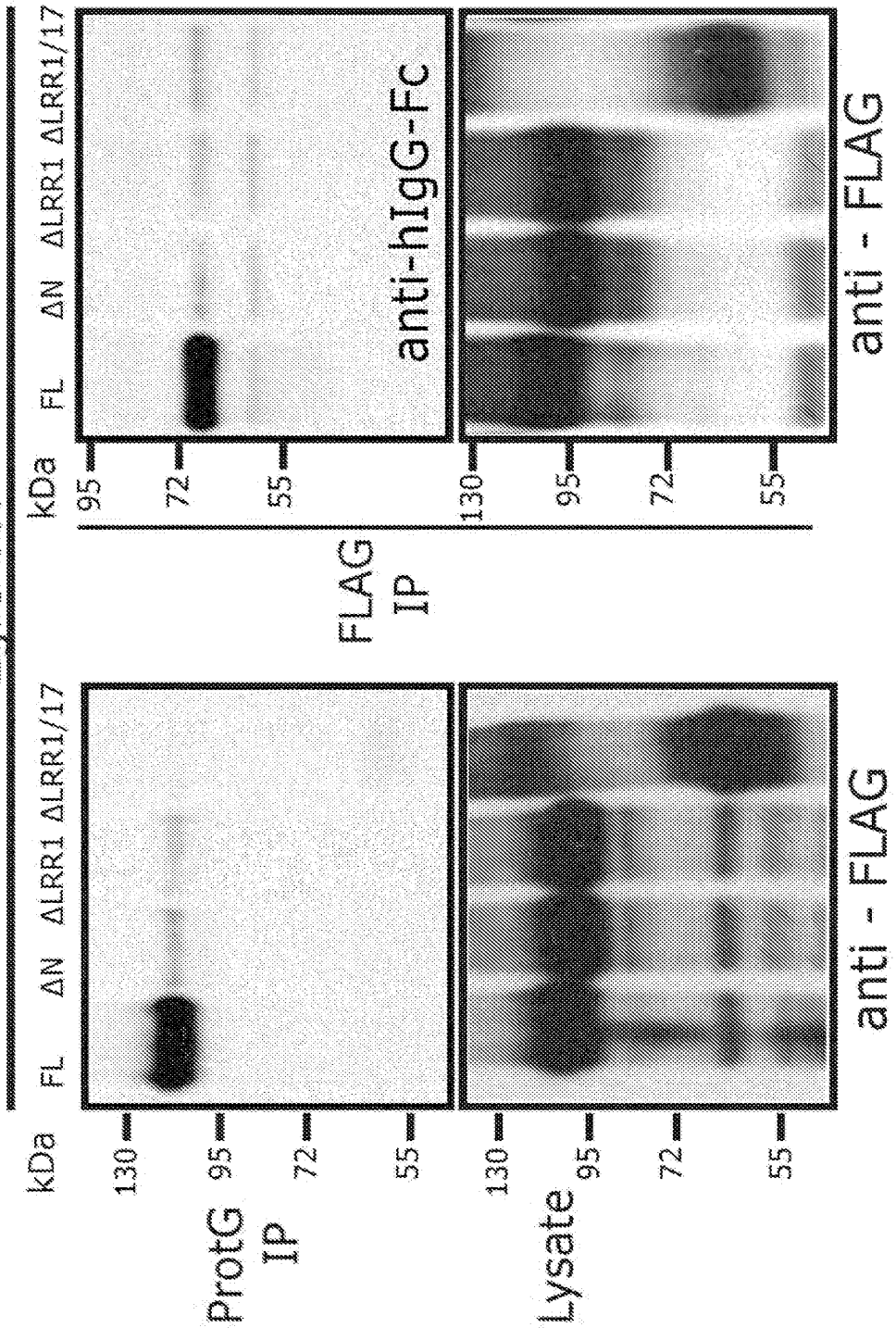

Lgr5-FH
kDa
FL
ΔN
ΔLRR1
ΔLRR1/17
130
95
72
55
ProtG IP
anti - FLAG
Lysate
FLAG IP
anti-hIgG-Fc

FIG. 15

K_D, k_d vs R_{max}

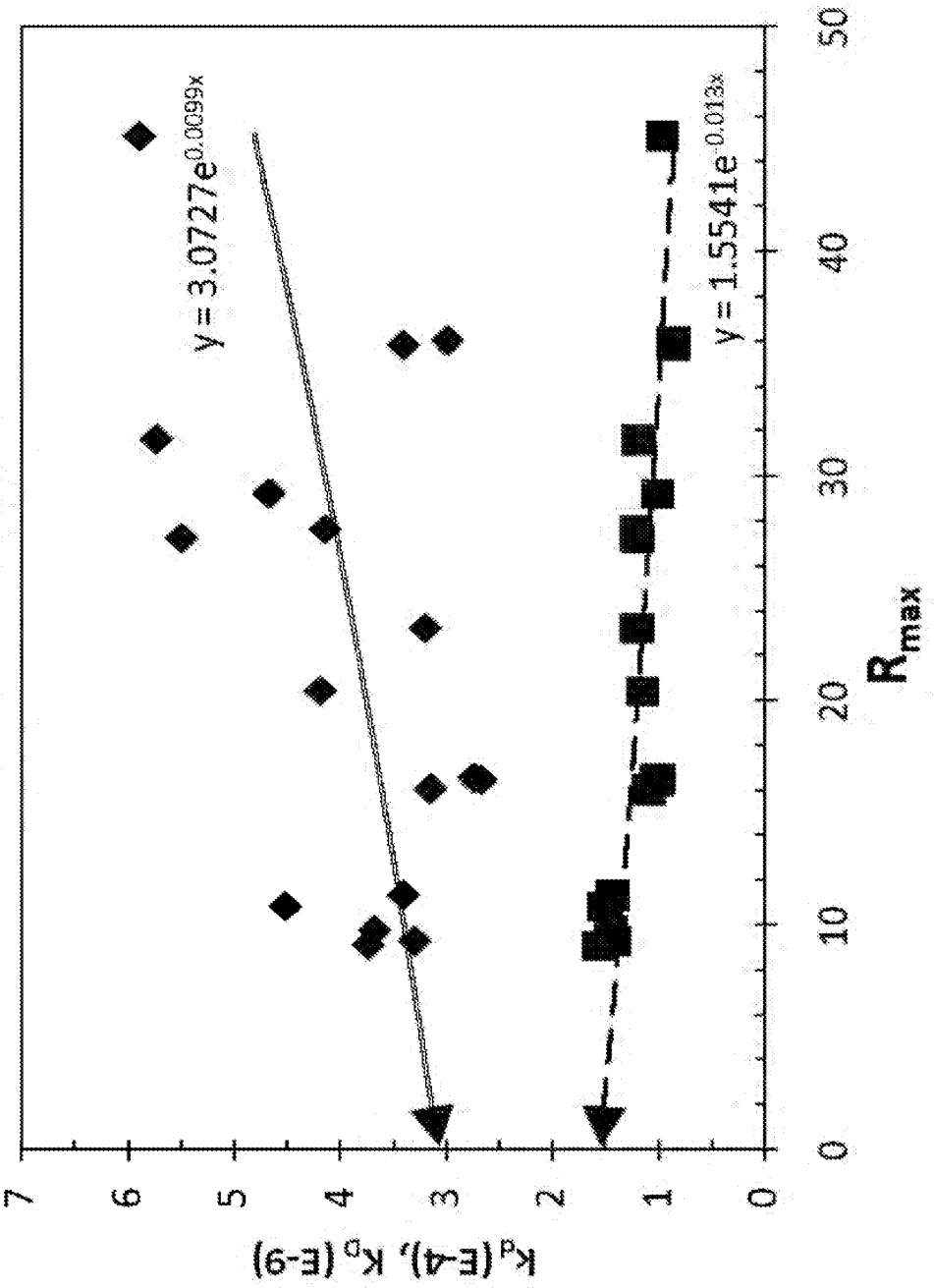
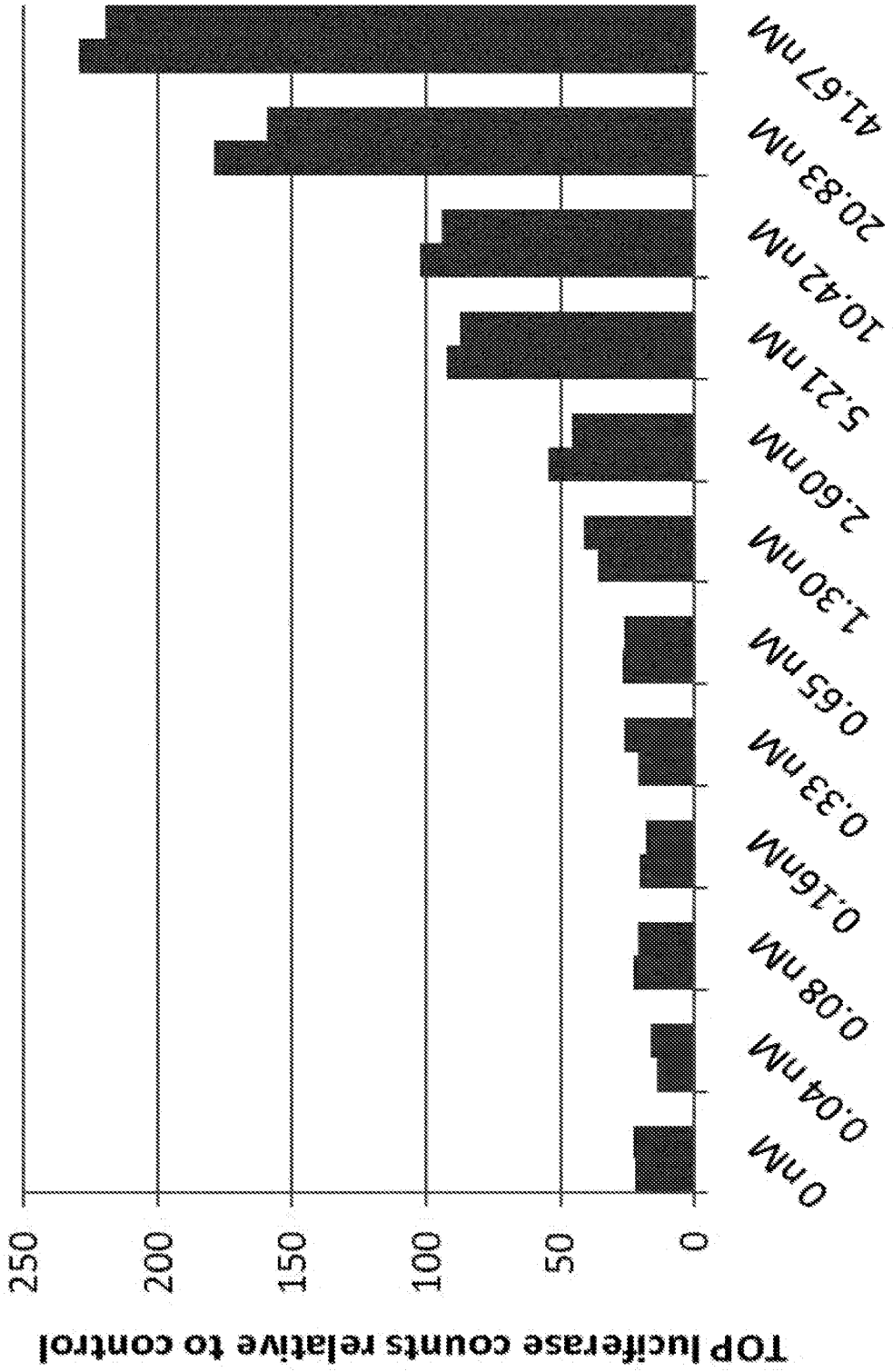


FIG. 16


TOP luciferase counts relative to control
250
200
150
100
50
0
0 nM
0.04 nM
0.08 nM
0.16nM
0.33 nM
0.65 nM
1.30 nM
2.60 nM
5.21 nM
10.42 nM
20.83 nM
41.67 nM

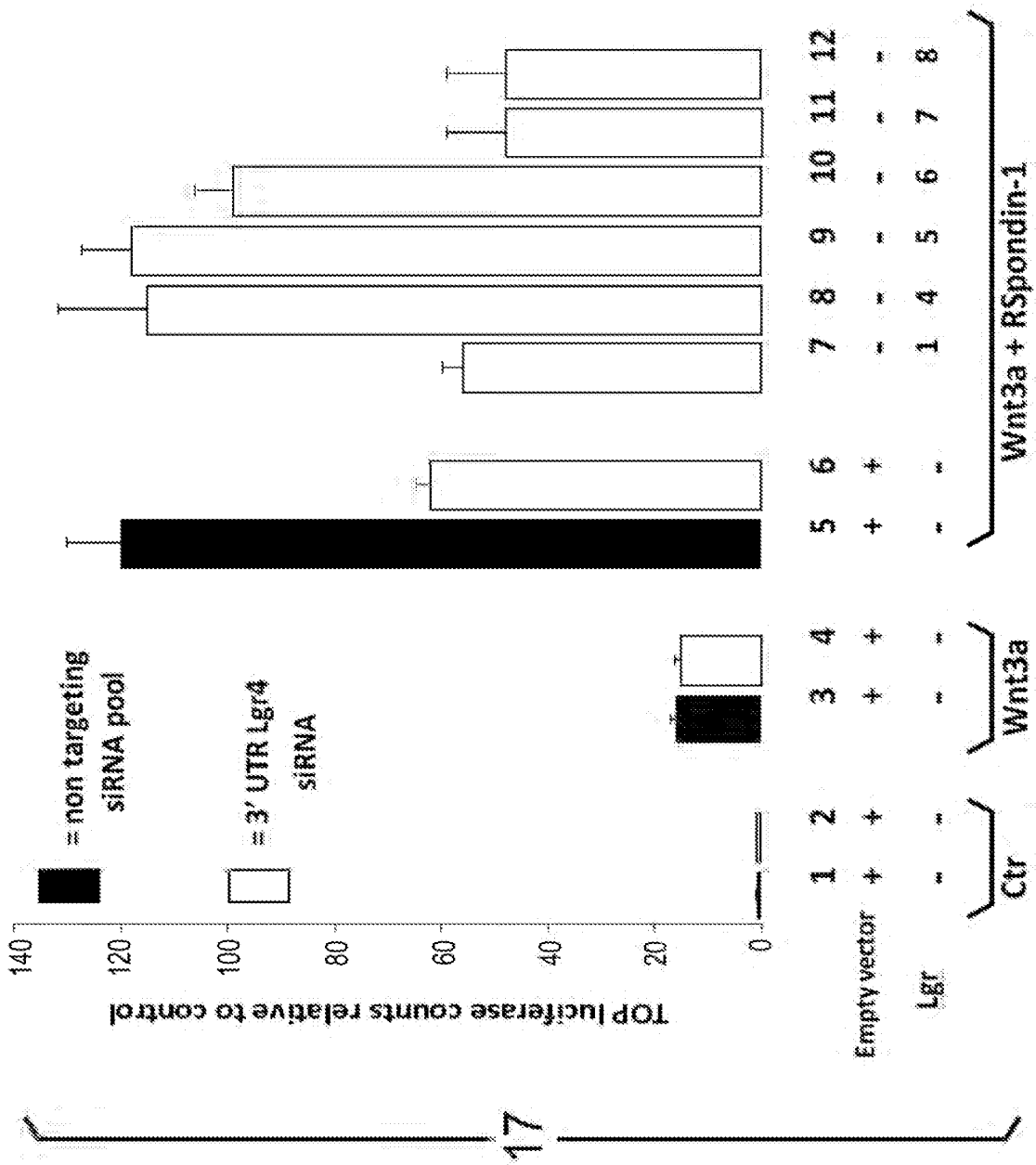

TOP luciferase counts relative to control
140
120
100
80
60
40
20
0
= non targeting siRNA pool
= 3' UTR Lgr4 siRNA
1 2 3 4 5 6 7 8 9 10 11 12
Empty vector + + + + + + - - - - - -
Lgr - - - - - - + + + + + +
Ctr
Wnt3a
Wnt3a + RSpondin-1

FIG. 17

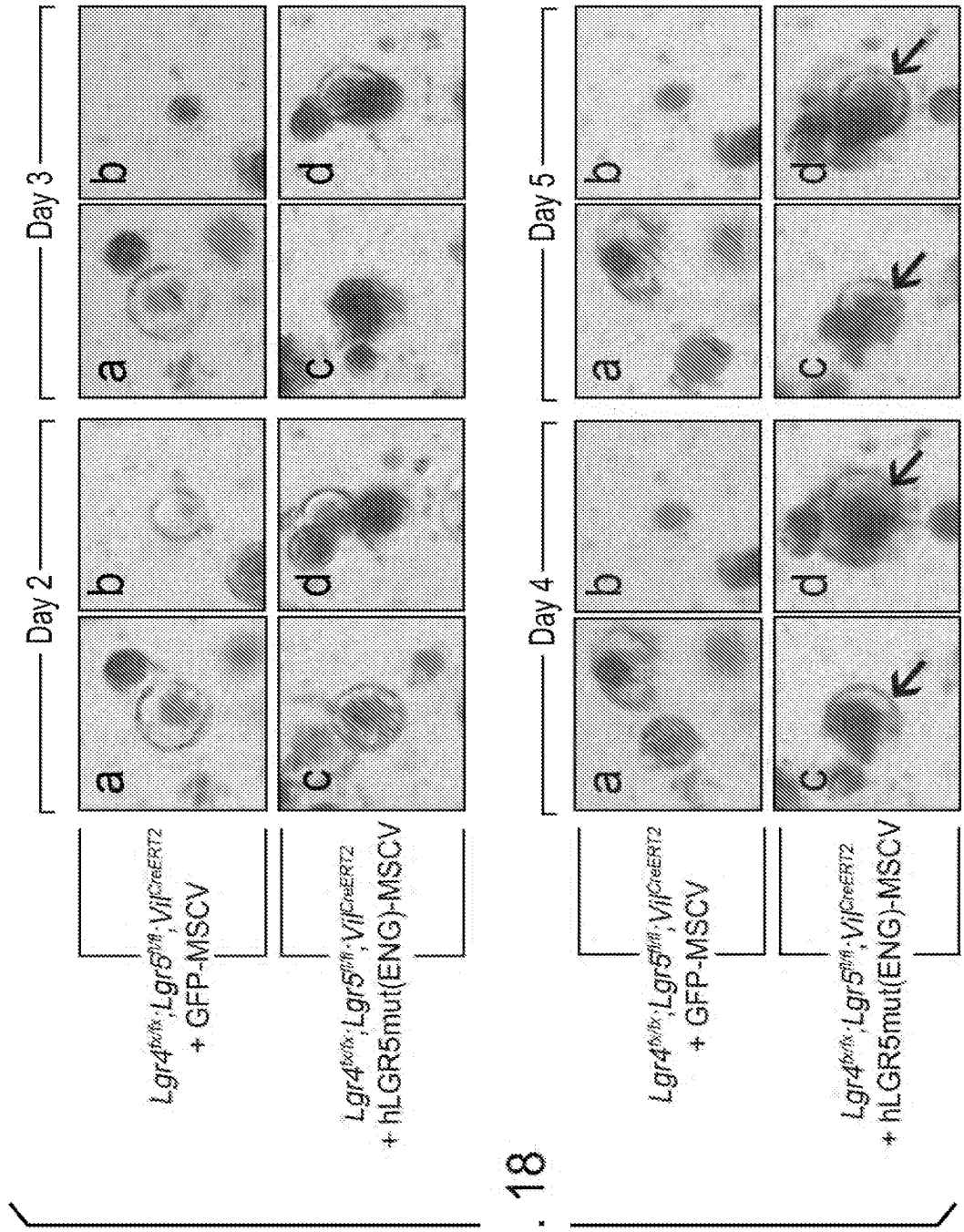

Day 2
Day 3
Day 4
Day 5
a
b
c
d
Lgr4fl/fl;Lgr5fl/fl;VilCreERT2
+ GFP-MSCV
Lgr4fl/fl;Lgr5fl/fl;VilCreERT2
+ hLGR5mut(ENG)-MSCV

FIG. 18

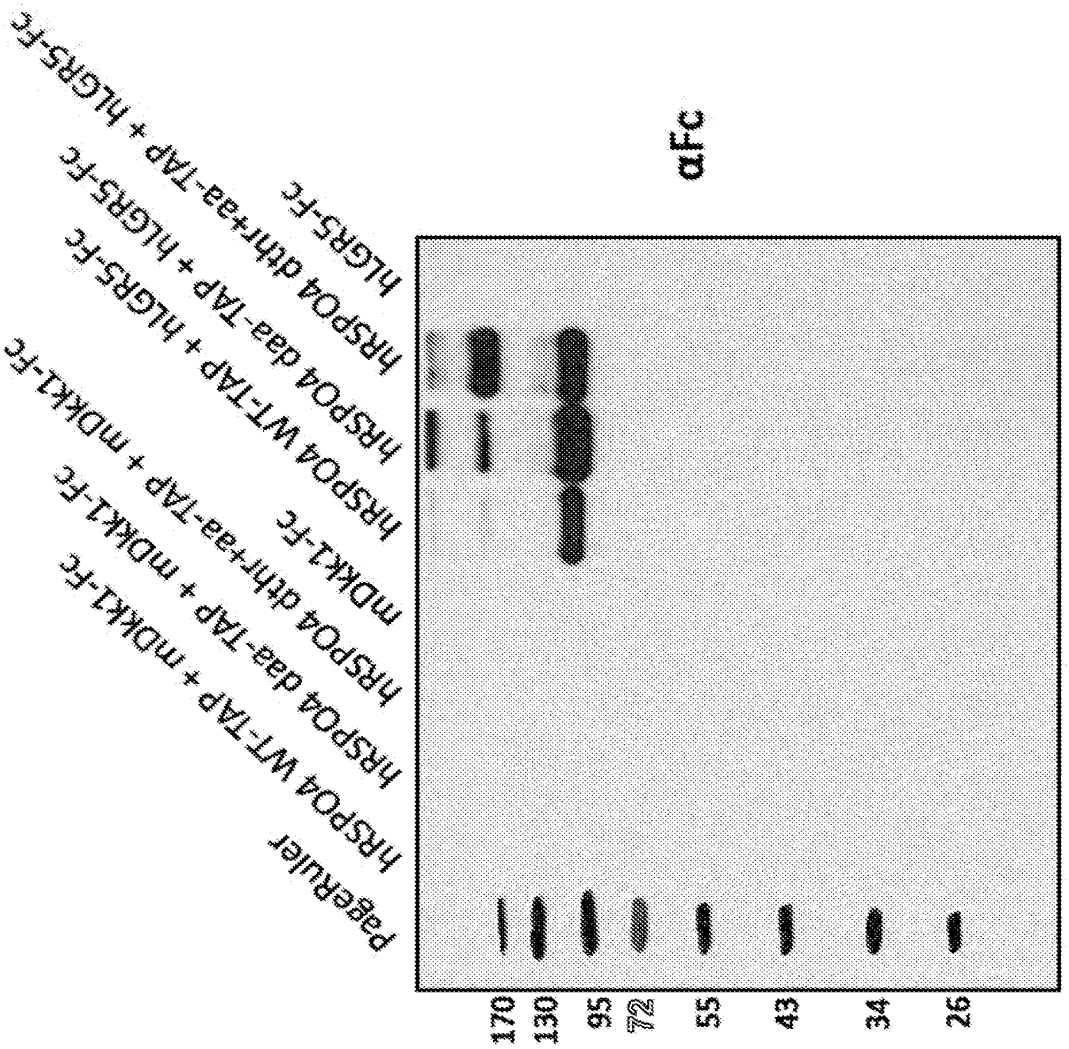

αFc
170
130
95
72
55
43
34
26
pageRuler
hRSPO4 WT-TAP + mDKK1-Fc
hRSPO4 daa-TAP + mDKK1-Fc
hRSPO4 dthr+aa-TAP + mDKK1-Fc
mDKK1-Fc
hRSPO4 WT-TAP + hLGR5-Fc
hRSPO4 daa-TAP + hLGR5-Fc
hRSPO4 dthr+aa-TAP + hLGR5-Fc
hLGR5-Fc

FIG. 19A

FIG. 19A (contd.)

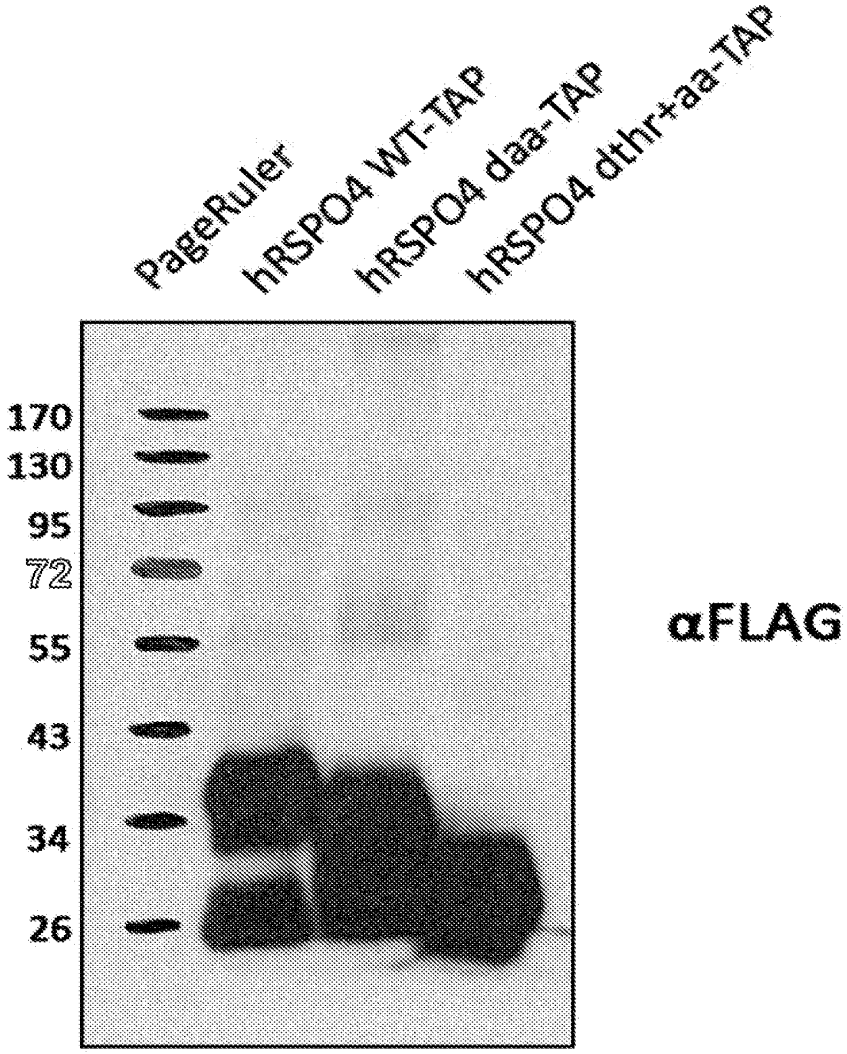
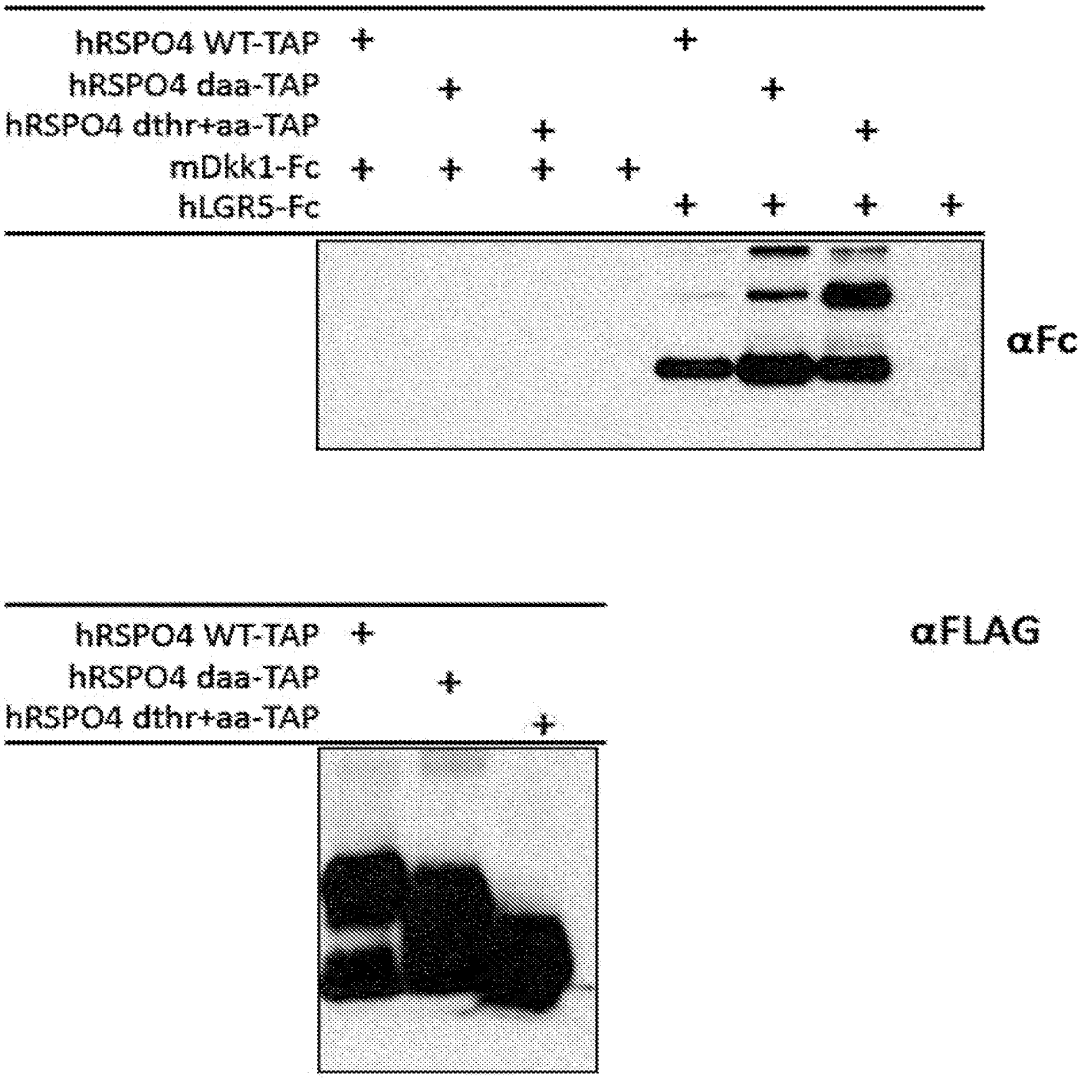


FIG. 19B


hRSPO4 WT-TAP + + +
hRSPO4 daa-TAP + + +
hRSPO4 dthr+aa-TAP + + +
mDkk1-Fc + + + +
hLGR5-Fc + + + +
αFc
hRSPO4 WT-TAP +
hRSPO4 daa-TAP +
hRSPO4 dthr+aa-TAP +
αFLAG

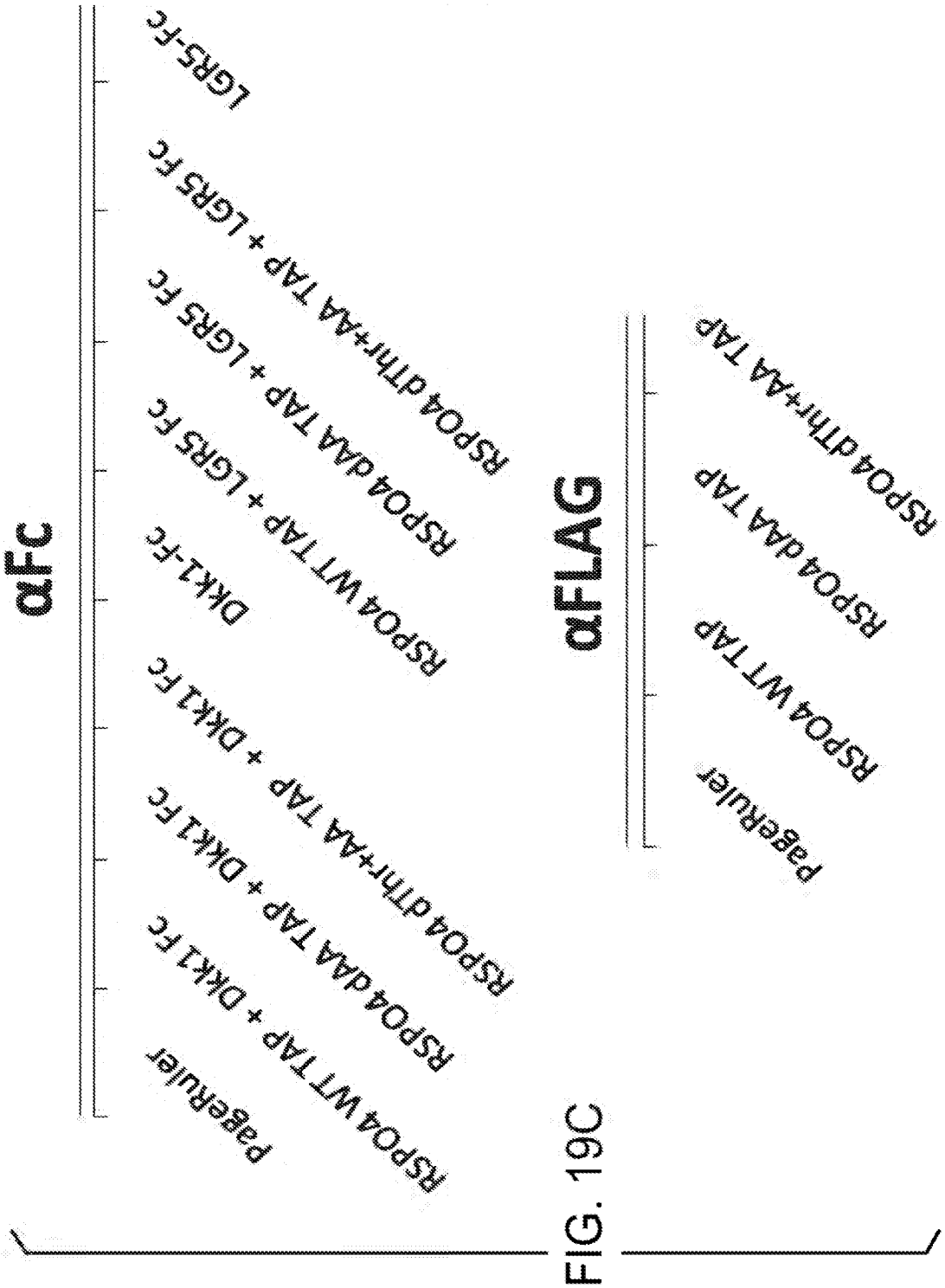

αFc
αFLAG
pageRuler
RSPO4 WT TAP + DKK1 Fc
RSPO4 dAA TAP + DKK1 Fc
RSPO4 dThr+AA TAP + DKK1 Fc
DKK1-Fc
RSPO4 WT TAP + LGR5 Fc
RSPO4 dAA TAP + LGR5 Fc
RSPO4 dThr+AA TAP + LGR5 Fc
LGR5-Fc
pageRuler
RSPO4 WT TAP
RSPO4 dAA TAP
RSPO4 dThr+AA TAP

FIG. 19C

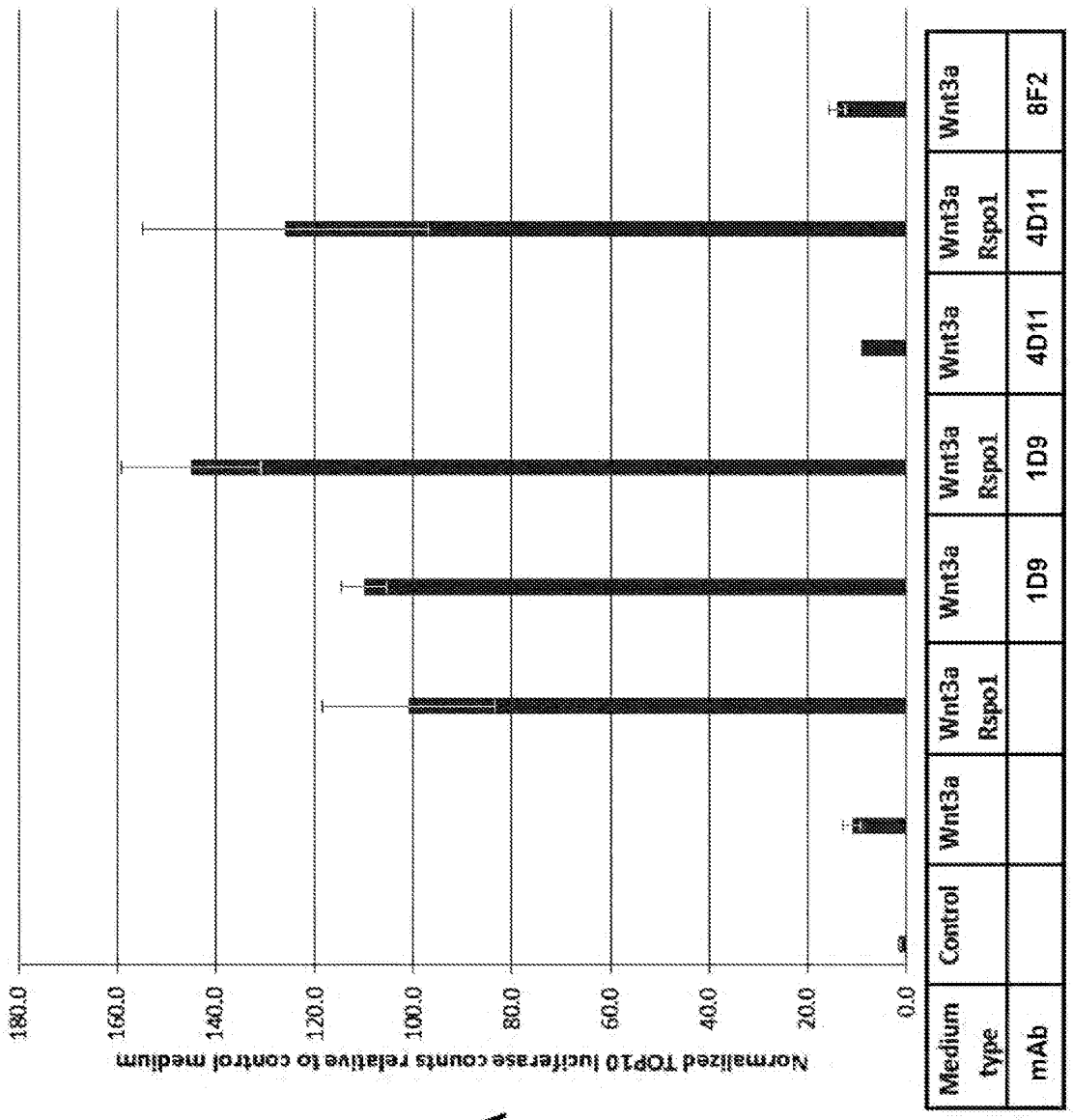

Normalized TOP10 luciferase counts relative to control medium
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Medium type
mAb
Control
Wnt3a
Wnt3a Rspo1
Wnt3a
1D9
Wnt3a Rspo1
1D9
Wnt3a
4D11
Wnt3a Rspo1
4D11
Wnt3a
8F2

FIG. 20A

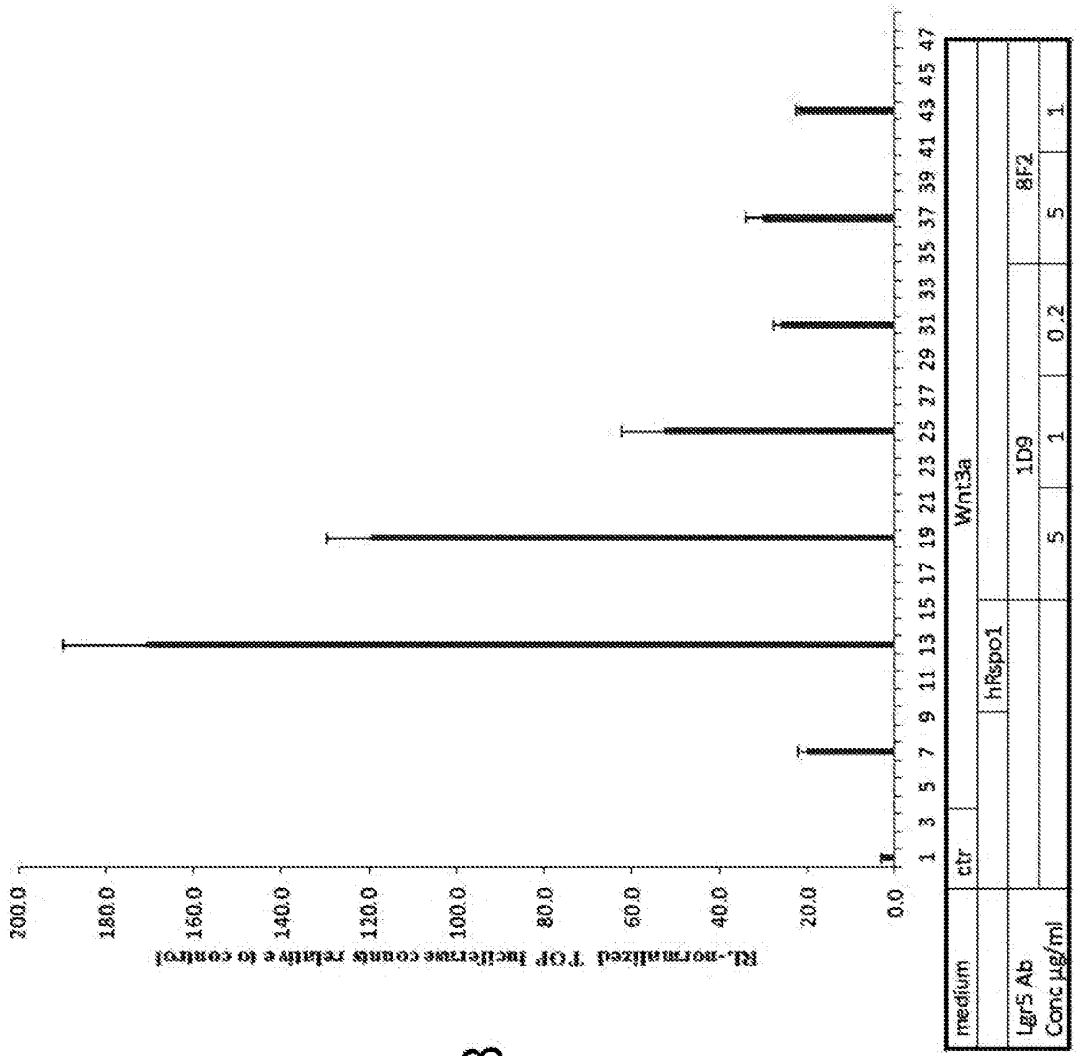
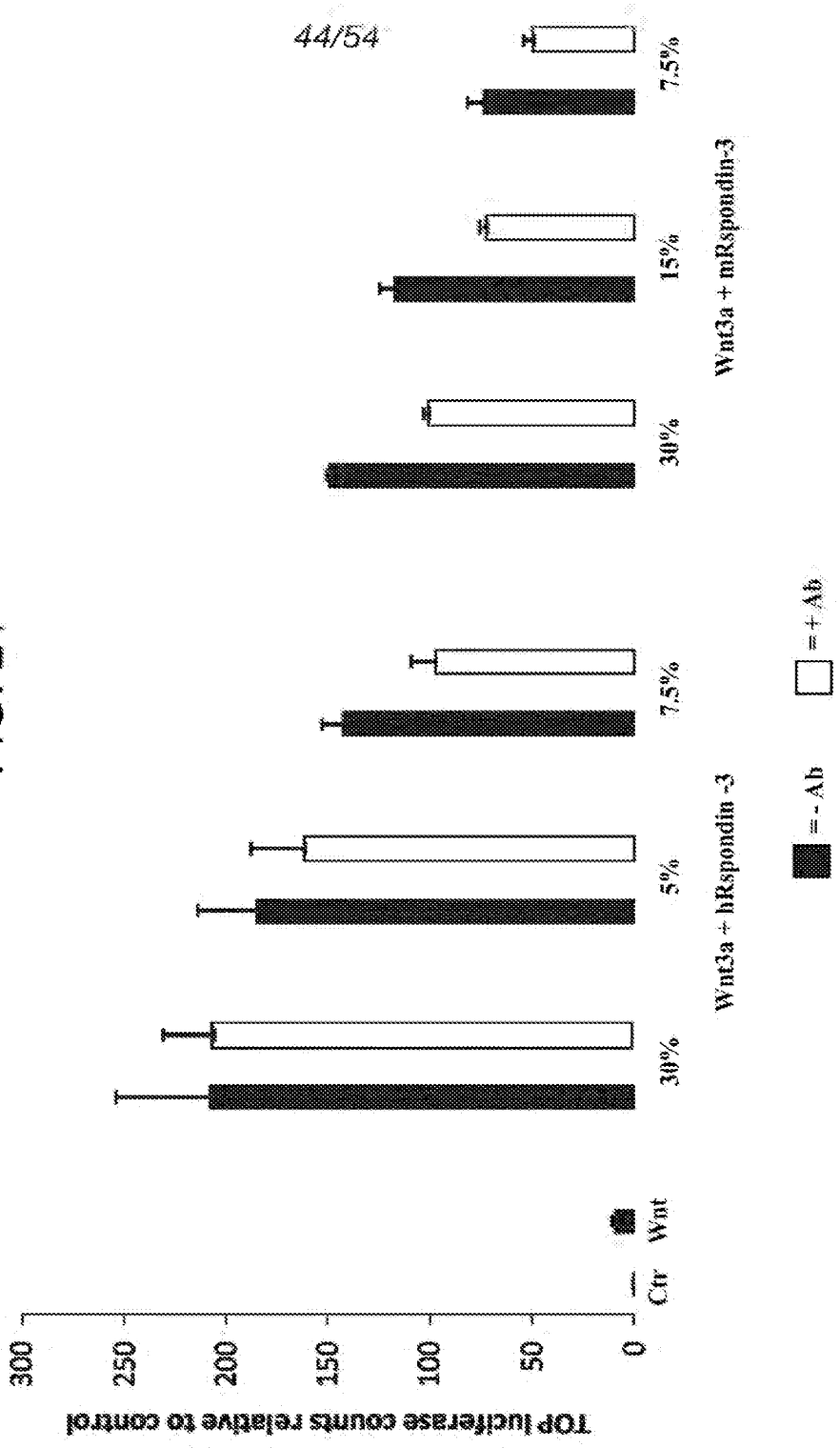

RL-normalized TOP luciferase counts relative to control
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
medium
ctr
hRspo1
Wnt3a
LgrS Ab Conc µg/ml
5
1
0.2
1D9
8F2
5
1

FIG. 20B

FIG. 21


TOP luciferase counts relative to control
300
250
200
150
100
50
0
— Ctr
Wnt
30%
5%
7.5%
Wnt3a + hRspondin -3
30%
15%
7.5%
Wnt3a + mRspondin-3
= - Ab
= + Ab

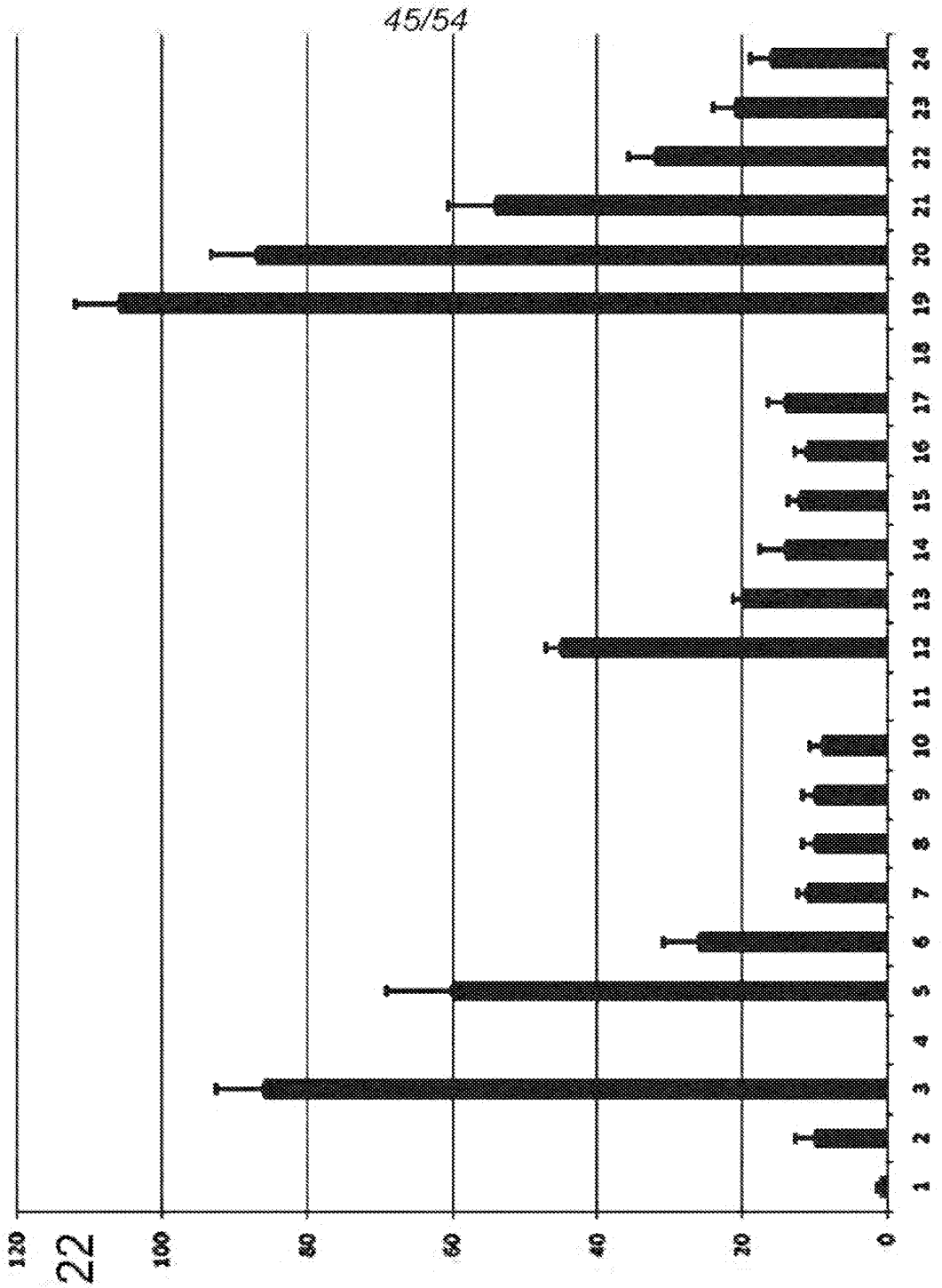

120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

FIG. 22

FIG. 23

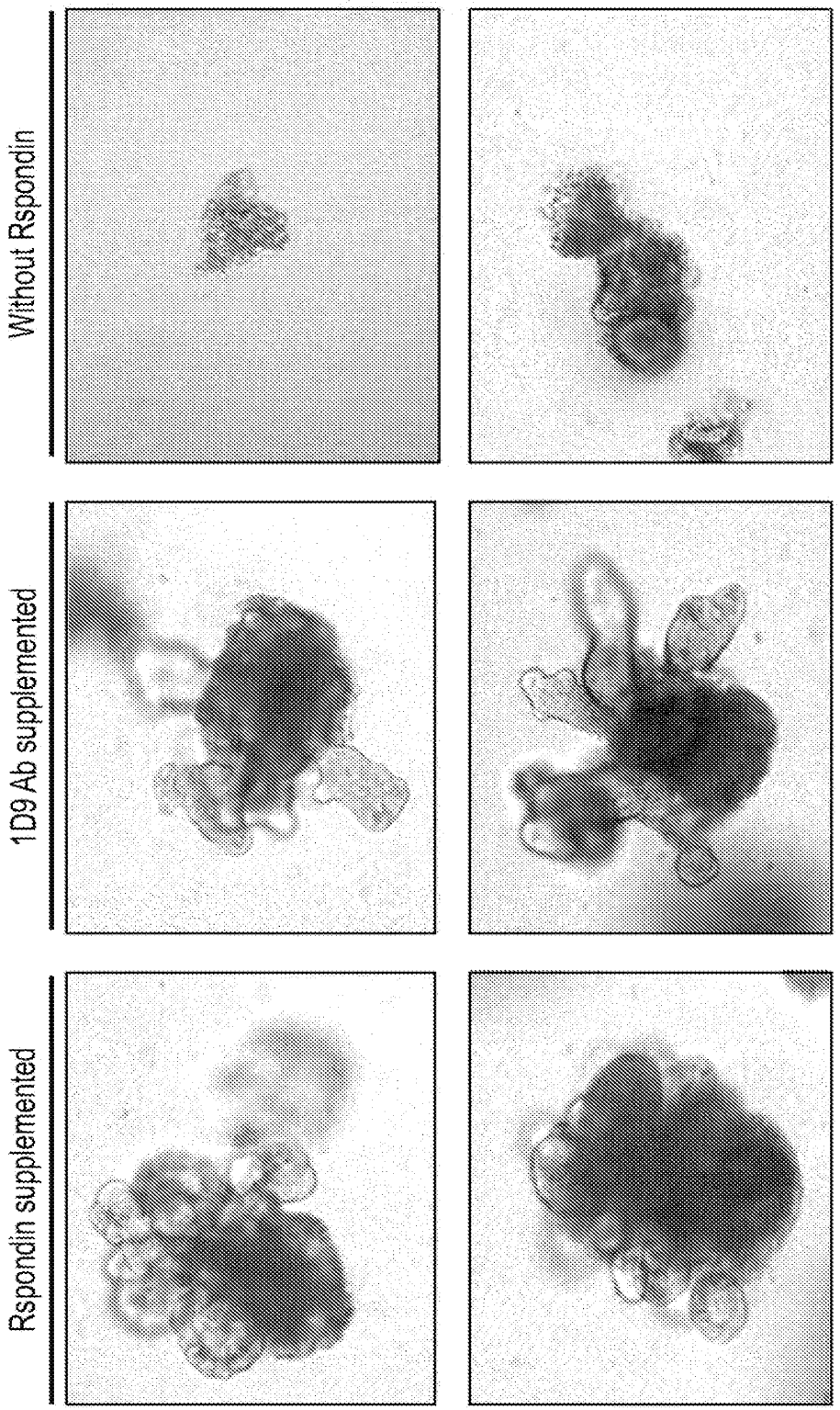
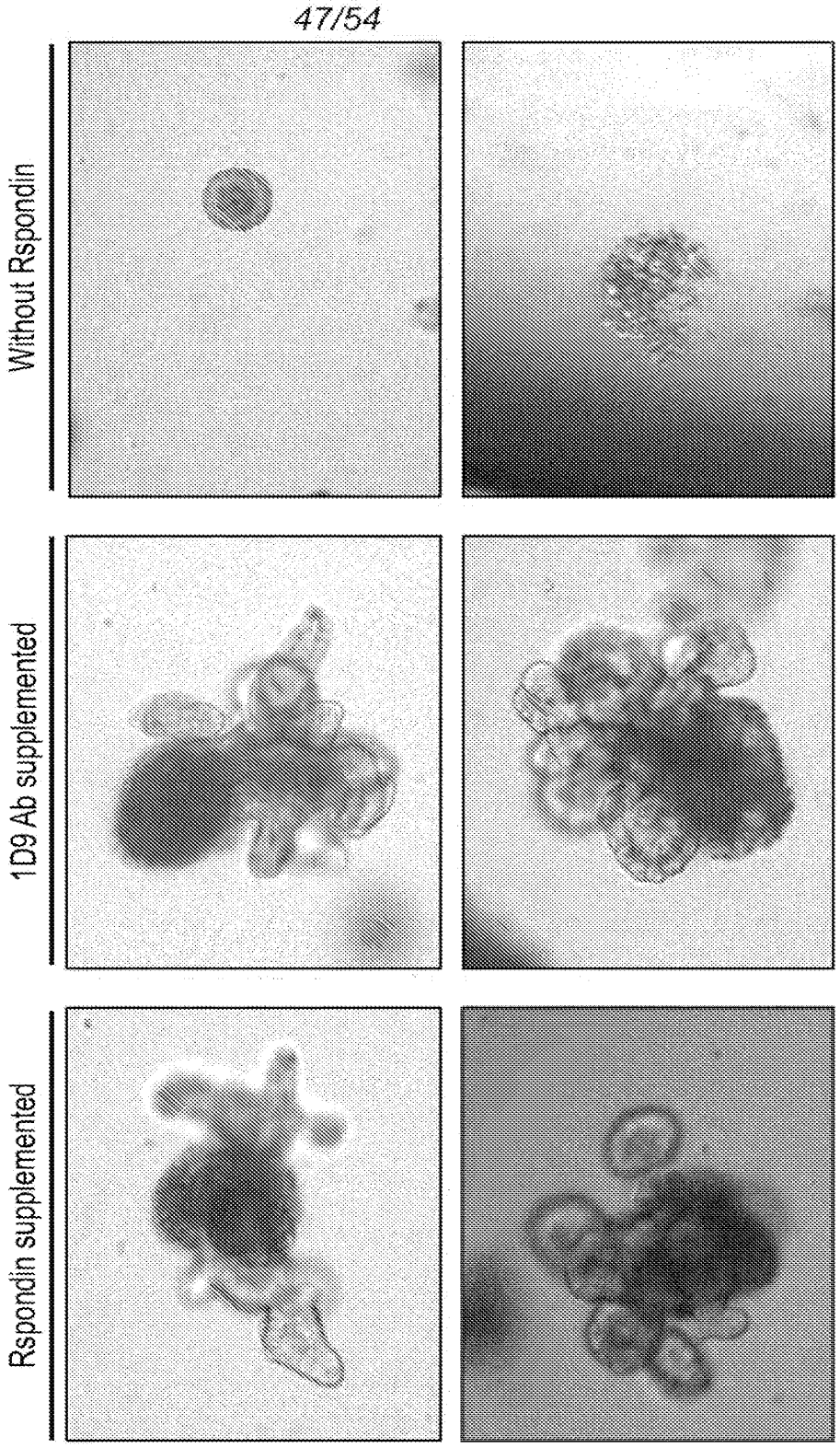

Without Rspondin
1D9 Ab supplemented
Rspondin supplemented

FIG. 23 (contd.)


Without Rspondin
1D9 Ab supplemented
Rspondin supplemented

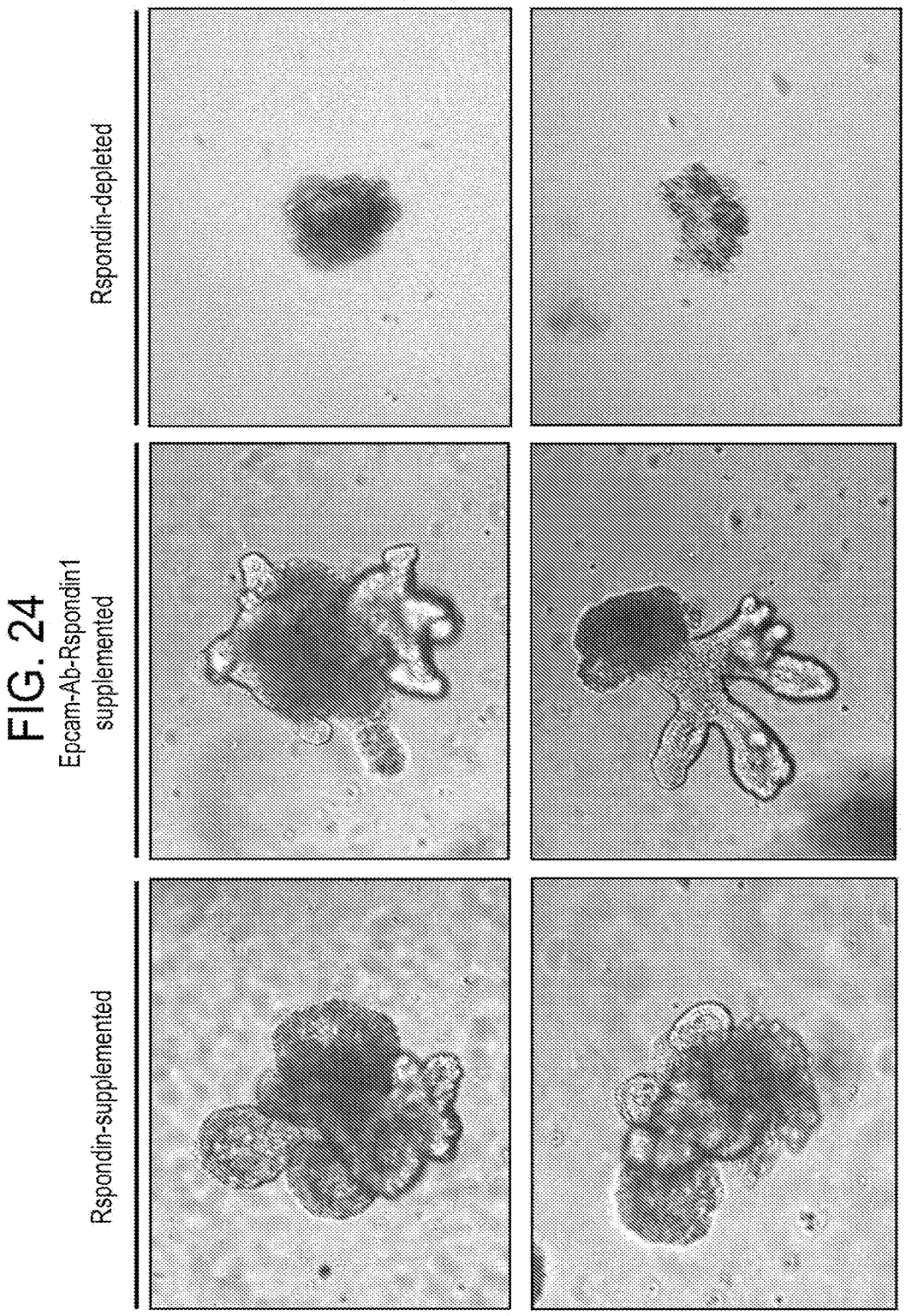

Rspondin-supplemented
Epcam-Ab-Rspondin1 supplemented
Rspondin-depleted

FIG. 24

FIG. 24 (contd.)

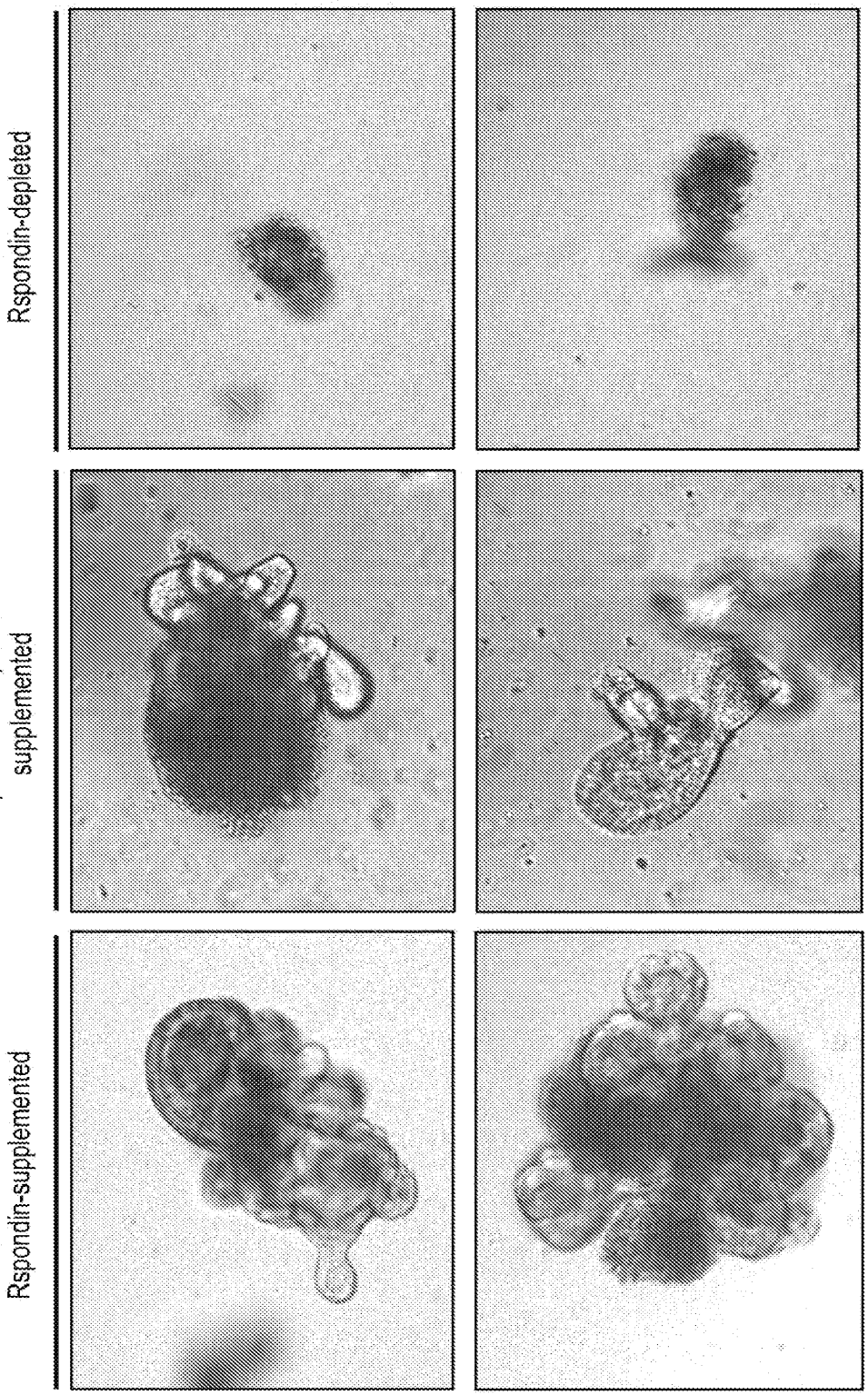

Rspondin-depleted
Epcam-Ab-Rspondin1 supplemented
Rspondin-supplemented

FIG. 25

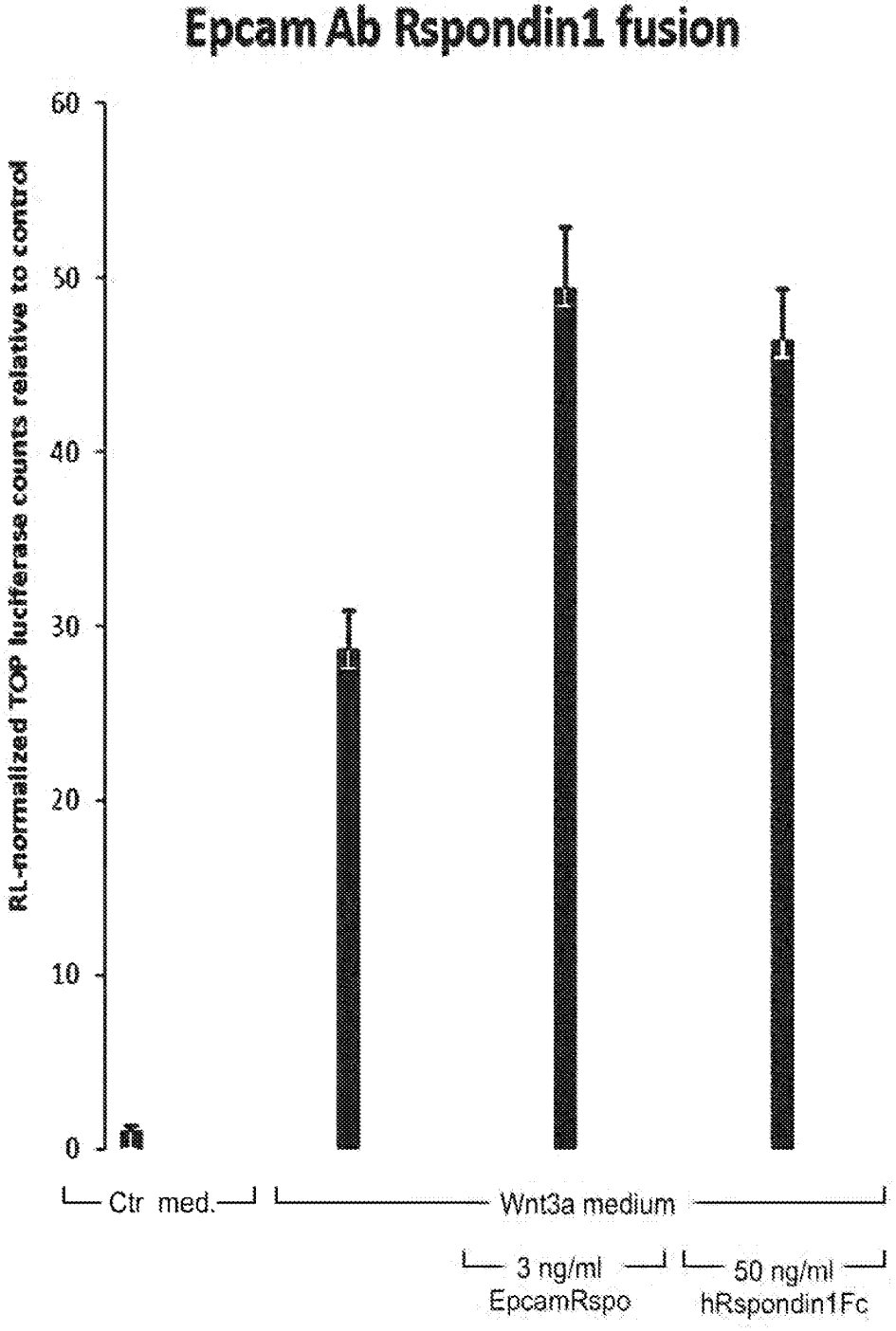

Epcam Ab Rspondin1 fusion
RL-normalized TOP luciferase counts relative to control
60
50
40
30
20
10
0
Ctr med.
Wnt3a medium
3 ng/ml
EpcamRspo
50 ng/ml
hRspondin1Fc

FIG. 26

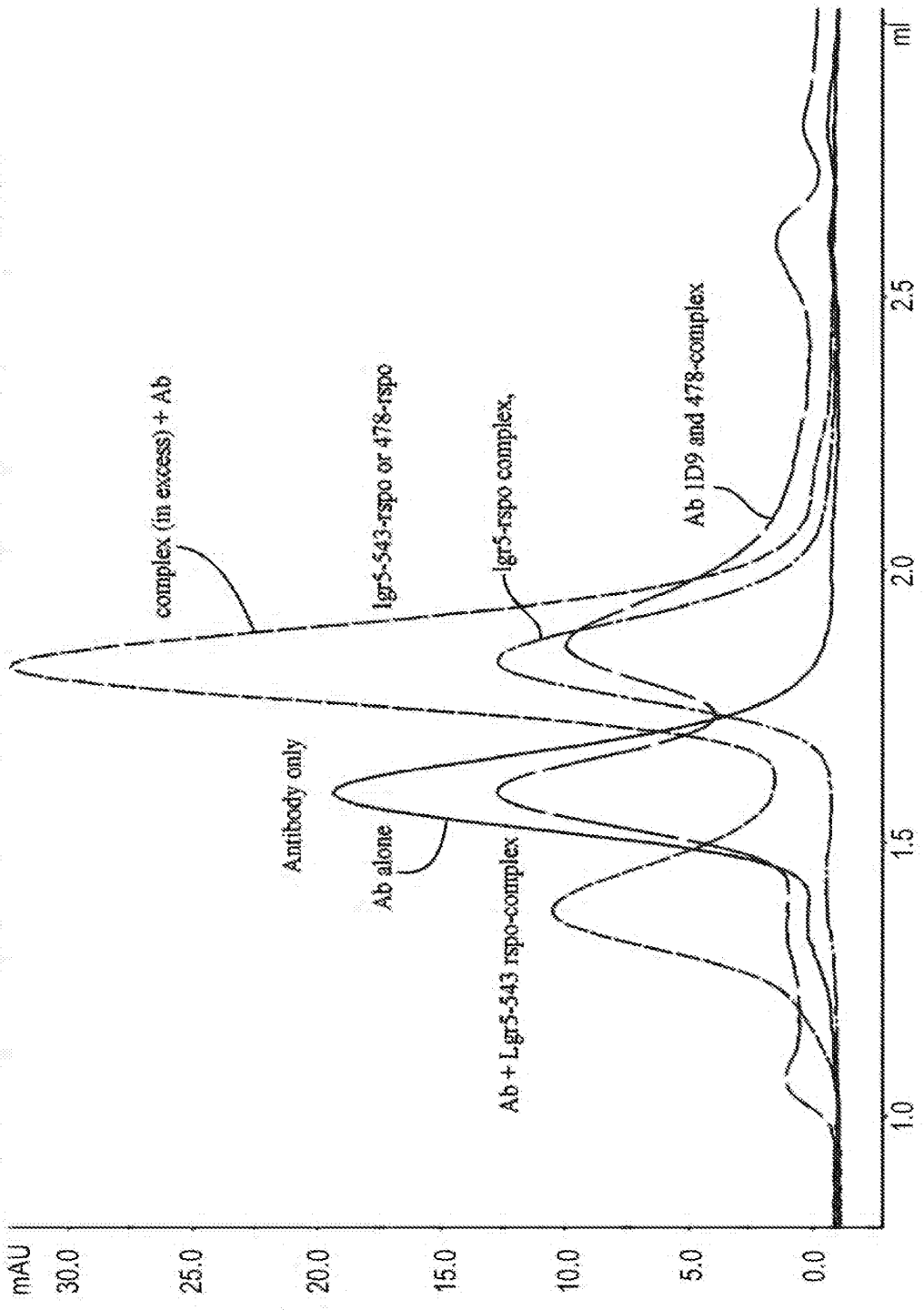

mAU
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1.0
1.5
2.0
2.5
ml
complex (in excess) + Ab
Antibody only
Ab alone
lgr5-543-rspo or 478-rspo
Ab + Lgr5-543 rspo-complex
lgr5-rspo complex,
Ab 1D9 and 478-complex

FIG. 27

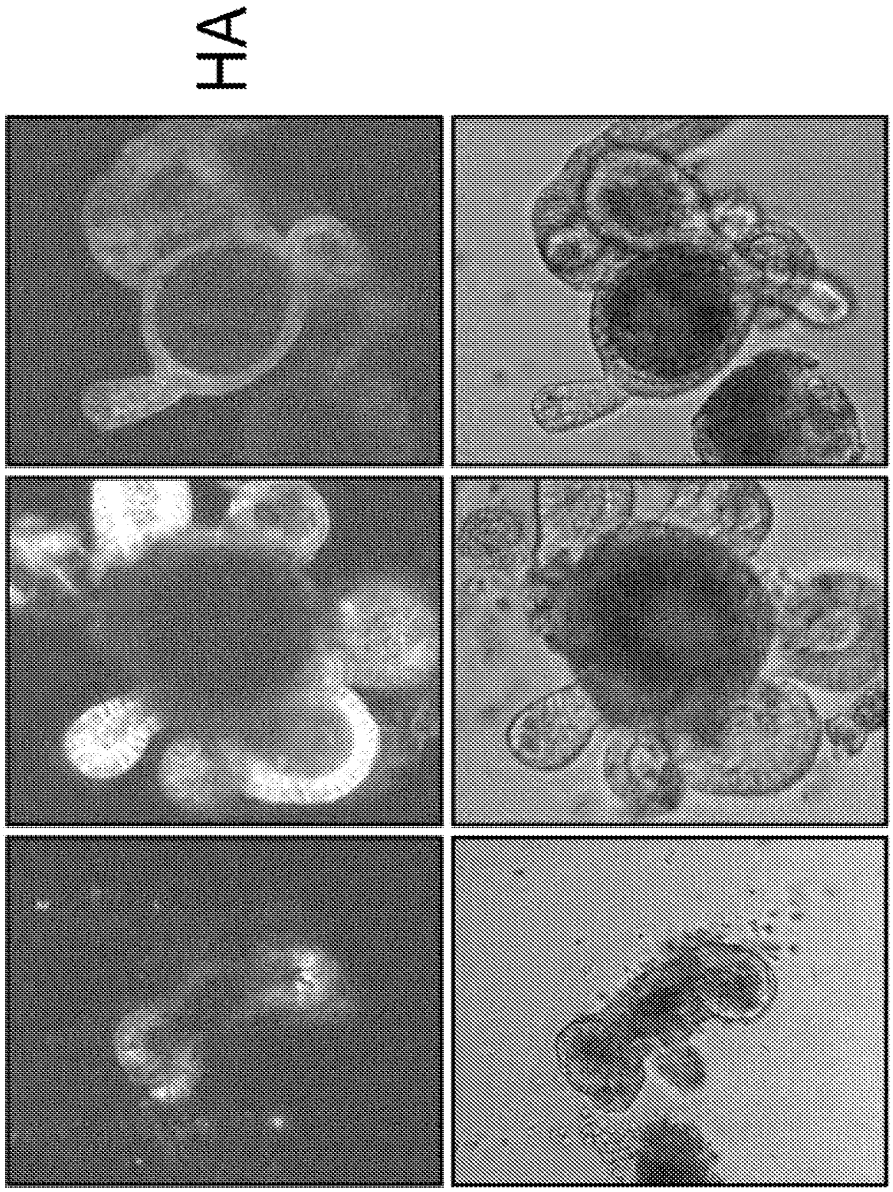


FIG. 28A

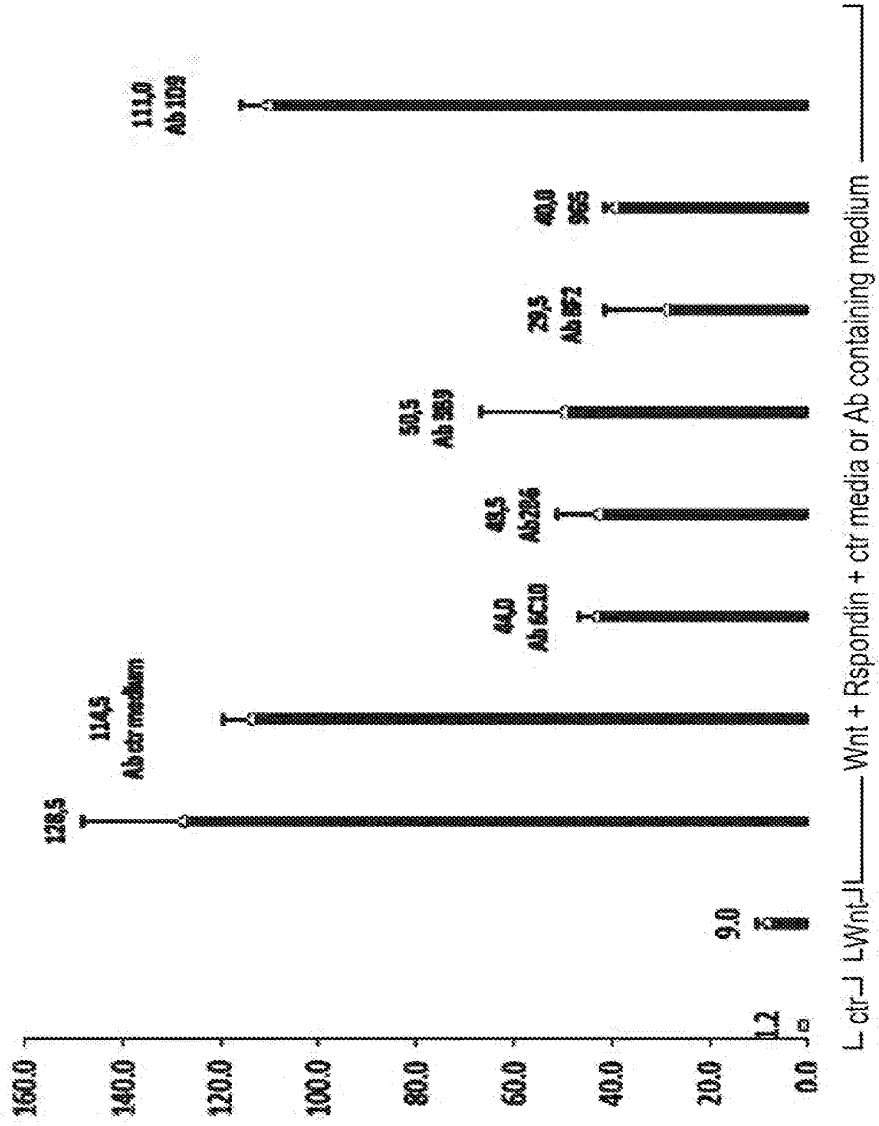
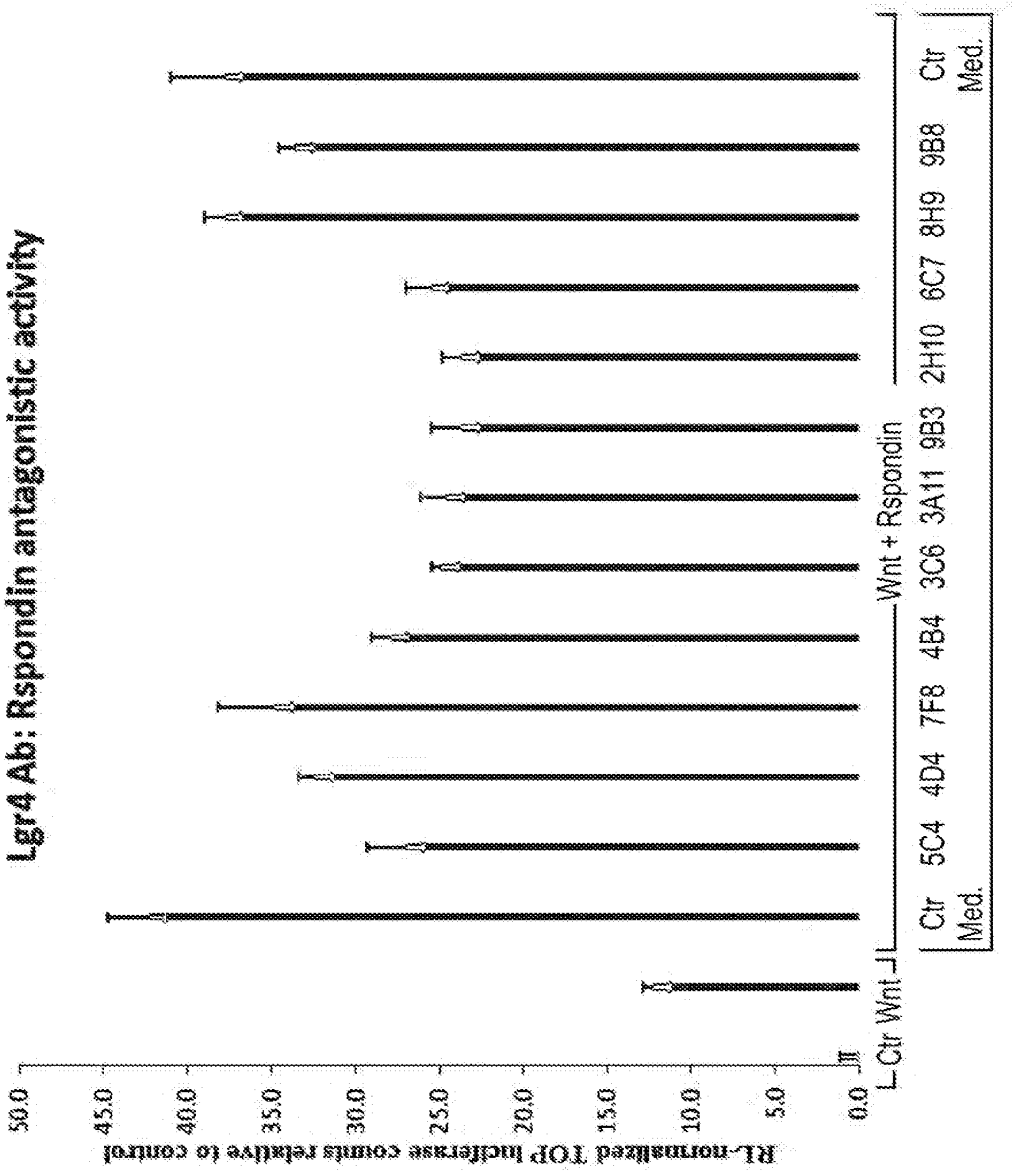

Lgr5 Ab: Rspondin antagonistic activity
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1.2
9.0
128.5
114.3
Ab ctr medium
111.0
Ab 1D9
40.0
Ab 6C10
43.3
Ab 2D6
50.3
Ab 8B9
29.3
Ab 9E7
40.0
9E6
L ctr
LWnt
Wnt + Rspondin + ctr media or Ab containing medium

FIG. 28B


Lgr4 Ab: Rspondin antagonistic activity
RL-normalized TOP luciferase counts relative to control
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Ctr
Wnt
Wnt + Rspondin
Ctr
5C4
4D4
7F8
4B4
3C6
3A11
9B3
2H10
6C7
8H9
9B8
Ctr
Med.
Med.

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(54) **Title**: COMPOSITIONS AND METHODS FOR TREATING RETINAL DISEASE

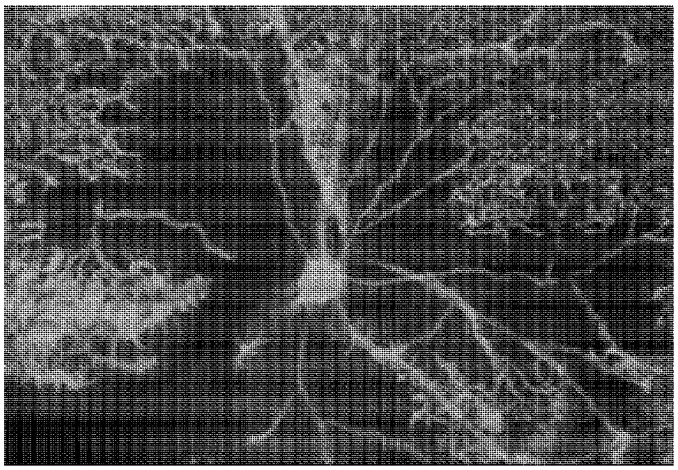


FIG. 1

(57) **Abstract**: The invention provides methods, compositions, and kits featuring Wnt signaling enhancing compounds for use in preventing or treating retinal disease.

WO 2014/143022 A1

COMPOSITIONS AND METHODS FOR TREATING RETINAL DISEASE

BACKGROUND OF THE INVENTION

The vertebrate retina is a light-sensitive layer of tissue that lines the inner surface of the eye and contains specialized photoreceptor cells referred to as rods and cones. These photoreceptor cells are connected to a network of nerve cells, which processes visual information received in the form of light striking the photoreceptor cells of the retina. This processed visual information is then sent to the brain for decoding into a final visual image.

The retina is susceptible to a variety of diseases, including: diabetic retinopathy, familial exudative vitreoretinopathy (FEVR), retinopathy of prematurity (ROP), Norries disease, and the like. For example, diabetic retinopathy is a retinal disease caused by complications of diabetes, which can eventually lead to blindness. Diabetic retinopathy is a prevalent form of retinal disease, which affects up to 80% of all patients who have had diabetes for 10 or more years. Diabetic retinopathy is the result of changes in the microvascular structure of the retina. The disease is generally considered to have two stages: an initial non-proliferative stage and a subsequent proliferative stage. Non-proliferative diabetic retinopathy is characterized by hyperglycemia-induced intramural pericyte death, as well as a thickening of the basement membrane that leads to incompetence of the vascular walls (e.g., capillary drop out). As the disease progresses, it enters an advanced proliferative stage in which the blood vessels of the retina begin to proliferate along the retina, as well as into the vitreous humor that fills the inside of the eye. Without proper treatment, these proliferating blood vessels can bleed (e.g., causing a macular edema), cloud vision, and ultimately destroy the retina. The primary treatment for diabetic retinopathy is laser treatment (e.g., laser photocoagulation, modified grid laser photocoagulation, pan retinal photocoagulation, etc.). Unfortunately, this type of treatment is not effective in curing diabetic retinopathy. Moreover, it has a significant disadvantage of causing a permanent loss of retinal tissue. In view of the foregoing, there is a clear need to develop compositions and methods for treating retinal diseases.

SUMMARY OF THE INVENTION

As described below, the present invention is based upon the discovery that agents that enhance Wnt signaling have the ability to treat and/or prevent capillary dropout, thereby treating

and/or preventing many forms of retinal disease. The invention features compositions and kits containing Wnt signaling activators. The invention also features methods for using these novel therapeutic molecules to treat a subject having, or at risk of having, a retinal disease associated with capillary dropout such as, for example, diabetic retinopathy, familial exudative vitreoretinopathy (FEVR), retinopathy of prematurity (ROP), or Norries disease.

In one aspect, the invention features a method of treating or preventing capillary dropout in a subject, wherein the method includes administering to the subject an agent that enhances Wnt signaling, thereby treating or preventing capillary dropout. In one embodiment, the subject has or is at risk of developing capillary dropout. In one embodiment, the subject has or is at risk of developing familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), Norries disease, branch retinal vein occlusion, or central retinal vein occlusion. In one embodiment, the subject has or is at risk of developing diabetic retinopathy. In one embodiment, the ocular capillaries of the subject are evaluated by fluorescein angiography (FA). In one embodiment, the agent is administered to the subject when an antecedent lesion is identified. In one embodiment, the antecedent lesion is located in a peripheral capillary. In one embodiment, the antecedent lesion is located in a posterior capillary.

In one aspect, the invention features a method of treating or preventing peripheral capillary dropout in a subject, wherein the method involves administering to the subject an agent that enhances Wnt signaling, thereby treating or preventing capillary dropout. In one embodiment, the subject has or is at risk of familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), Norries disease, branch retinal vein occlusion, or central retinal vein occlusion. In one embodiment, the subject has or is at risk of developing diabetic retinopathy. In one embodiment, the ocular capillaries of the subject are evaluated by fluorescein angiography (FA). In one embodiment, the agent is administered to the subject when an antecedent lesion is identified in a peripheral capillary. In one embodiment, the method reduces capillary loss. In one embodiment, the method reduces capillary occlusion. In one embodiment, the method enhances capillary formation. In one embodiment, In one embodiment,the method enhances retinal function. In one embodiment, the method enhances b-wave response, substantial oscillatory potential, or visual improvement. In one embodiment, the method reduces the symptoms associated with capillary dropout. In one embodiment, the agent enhances the activity of a frizzled-4 transmembrane receptor (Fzd4). In one embodiment, the

agent is an agonist of Fzd4, Low-density lipoprotein receptor-related protein 5 (LRP5), Low-density lipoprotein receptor-related protein 6 (LRP6), or tetraspanin-12 (Tspan12). In one embodiment, the agent is a small molecule, a nucleic acid, a peptide, or a peptide mimetic. In one embodiment, the agent is an antibody or antibody fragment. In one embodiment, the small molecule is 2-amino-4-[3,4-(methylenedioxy)benzyl-amino]-6-(3-methoxyphenyl)pyrimidine. In one embodiment, the antibody is a polyclonal antibody. In one embodiment, the antibody is a monoclonal antibody. In one embodiment, the antibody or antibody fragment is humanized. In one embodiment, the agent is a ligand of Fzd4. In one embodiment, the ligand is Norrin, an active fragment of Norrin, or an active mutant of Norrin. In one embodiment, the agent is a nucleic acid encoding Norrin or a fragment thereof.

In one aspect, the invention features a method of treating or preventing capillary dropout in a subject having or at risk of developing diabetic retinopathy, wherein the method comprises administering Norrin or a fragment thereof to the subject, thereby treating or preventing capillary dropout. In one embodiment, the method further comprises administering at least one additional agent to treat capillary dropout. In one embodiment, the at least one additional agent is an anti-inflammatory, a steroid, anti-VEGF, or a caspase. In one embodiment, the method further comprises treating the patient with laser therapy. In one embodiment, the agent is administered systemically or locally. In one embodiment, the agent is administered orally, parenterally, or topically. In one embodiment, the agent is administered by intravitreal or periocular injection. In one embodiment, the subject is a mammal. In one embodiment, the subject is human.

In one aspect, the invention features a pharmaceutical composition comprising an agent that treats capillary dropout. In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout enhances the activity of a frizzled-4 transmembrane receptor (Fzd4). In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout and is an agonist of Fzd4, Low-density lipoprotein receptor-related protein 5 (LRP5), or tetraspanin-12 (Tspan12). In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout and is a small molecule, a nucleic acid, a peptide, or a peptide mimetic. In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout that is an antibody or antibody fragment. In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout and is a polyclonal antibody. In one embodiment, the pharmaceutical composition includes an agent that treats

capillary dropout and is a monoclonal antibody. In one embodiment, the pharmaceutical composition includes an antibody or antibody fragment that is humanized. In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout and is a ligand of Fzd4. In one embodiment, the pharmaceutical composition includes a ligand that is Norrin or a fragment thereof. In one embodiment, the pharmaceutical composition includes an agent that treats capillary dropout and is a nucleic acid encoding Norrin or a fragment thereof. In one embodiment, the pharmaceutical composition includes at least one additional agent to treat capillary dropout. In one embodiment, the pharmaceutical composition further includes a pharmaceutically acceptable carrier, diluent, or excipient.

In one aspect, the invention features a kit comprising an agent that treats capillary dropout. In one embodiment, the agent that treats capillary dropout enhances the activity of a frizzled-4 transmembrane receptor (Fzd4). In one embodiment, the agent that treats capillary dropout is an agonist of Fzd4, Low-density lipoprotein receptor-related protein 5 (LRP5), or tetraspanin-12 (Tspan12). In one embodiment, the agent that treats capillary dropout is a small molecule, a nucleic acid, a peptide, or a peptide mimetic. In one embodiment, the agent that treats capillary dropout is an antibody or antibody fragment. In one embodiment, the agent that treats capillary dropout is a polyclonal antibody. In one embodiment, the agent that treats capillary dropout is a monoclonal antibody. In one embodiment, the antibody or antibody fragment is humanized. In one embodiment, the agent that treats capillary dropout is a ligand of Fzd4. In one embodiment, the ligand is Norrin or a fragment thereof. In one embodiment, the agent that treats capillary dropout is a nucleic acid encoding Norrin or a fragment thereof. In one embodiment, the kit further includes at least one additional agent to treat capillary dropout. In one embodiment, the kit further comprises instructions for using the agent that treats capillary dropout as described above.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

By "activate" is meant an increase in activity, level, or other measurable parameter relative to a reference (i.e., an untreated control). Such activation can be by about 10%, 25%, 50%, 75% or more.

"Administering" is defined herein as a means of providing an agent or a composition containing the agent to a subject in a manner that results in the agent being inside the subject's body. Such an administration can be by any route including, without limitation, oral, transdermal (e.g., vagina, rectum, oral mucosa), by injection (e.g., subcutaneous, intravenous, parenterally, intraperitoneally, intrathecal, intraocular), by inhalation (e.g., oral or nasal), or topical (e.g., eyedrops, cream, etc.). Pharmaceutical preparations are, of course, given by forms suitable for each administration route.

By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule or polypeptide, or fragments thereof.

By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% change in expression levels, preferably a 25% change, more preferably a 40% change, and most preferably a 50% or greater change in expression levels.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

By "analog" is meant a molecule that is not identical, but has analogous functional or structural features. For example, a polypeptide analog retains the biological activity of a corresponding naturally-occurring polypeptide, while having certain biochemical modifications that enhance the analog's function relative to a naturally occurring polypeptide. Such biochemical modifications could increase the analog's protease resistance, membrane permeability, or half-life, without altering, for example, ligand binding. An analog may include an unnatural amino acid.

By "antecedent lesion" is meant an implied cellular change due to damage of endothelial cells. For example, an antecedent lesion may be indicated by a retinal vessel that has indistinct margins in an image, and/or in which the vessel diameter is increased with

indistinct margins in a fundus image where the rest of the vessels maintain a crisp architecture.

As used herein, the term "antibody" means not only intact antibody molecules, but also fragments of antibody molecules that retain immunogen-binding ability. Such fragments are also well known in the art and are regularly employed both in vitro and in vivo. Accordingly, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments F(ab')2, and Fab. F(ab')2, and Fab fragments that lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non specific tissue binding of an intact antibody (Wahl et al., J. Nucl. Med. 24:316 325 (1983). The antibodies of the invention comprise whole native antibodies, bispecific antibodies; chimeric antibodies; Fab, Fab', single chain V region fragments (scFv), fusion polypeptides, and unconventional antibodies. Unconventional antibodies include, but are not limited to, nanobodies, linear antibodies (Zapata et al., Protein Eng. 8(10): 1057-1062,1995), single domain antibodies, single chain antibodies, and antibodies having multiple valencies (e.g., diabodies, tribodies, tetrabodies, and pentabodies). Nanobodies are the smallest fragments of naturally occurring heavy-chain antibodies that have evolved to be fully functional in the absence of a light chain. Nanobodies have the affinity and specificity of conventional antibodies although they are only half of the size of a single chain Fv fragment. The consequence of this unique structure, combined with their extreme stability and a high degree of homology with human antibody frameworks, is that nanobodies can bind therapeutic targets not accessible to conventional antibodies. Recombinant antibody fragments with multiple valencies provide high binding avidity and unique targeting specificity to cancer cells. These multimeric scFvs (e.g., diabodies, tetrabodies) offer an improvement over the parent antibody since small molecules of ~60-100kDa in size provide faster blood clearance and rapid tissue uptake. See Power et al., (Generation of recombinant multimeric antibody fragments for tumor diagnosis and therapy. Methods Mol Biol, 207, 335-50, 2003); and Wu et al. (Anti-carcinoembryonic antigen (CEA) diabody for rapid tumor targeting and imaging. Tumor Targeting, 4, 47-58, 1999).

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is

recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

As used herein, the term "capillary drop out" means that the small capillary vessels of the retina, which fill the capillary beds of the retina bridging the spaces between arteries and veins, become damaged by intravascular changes that lead to endothelial cell destruction. Consequently, the vessel elements are destroyed by apoptotic destruction and no longer able to nourish the retina. Such vessels are not visualized by angiography. The term "posterior capillary drop out" means that the foveal area of the macula is involved in capillary loss. The term "peripheral capillary drop out" means that areas other than the posterior capillary area are involved in capillary loss.

By "control" is meant a standard or reference condition.

The term "derivative" means a pharmaceutically active compound with equivalent or near equivalent physiological functionality to a given agent (e.g., a Wnt signaling activator or inhibitor). As used herein, the term "derivative" includes any pharmaceutically acceptable salt, ether, ester, prodrug, solvate, stereoisomer including enantiomer, diastereomer or stereoisomerically enriched or racemic mixture, and any other compound which upon administration to the recipient, is capable of providing (directly or indirectly) such a compound or an antivirally active metabolite or residue thereof.

"Detect" refers to identifying the presence, absence or amount of the analyte to be detected.

By "detectable label" is meant a composition that when linked to a molecule of interest renders the latter detectable, via spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes (for example, as commonly used in an ELISA), biotin, digoxigenin, or haptens.

By "effective amount" is meant the amount required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject.

Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

By "enhances" or "increases" is meant a positive alteration of at least about 10%, 25%, 50%, 75%, or 100% relative to a reference.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

By "fuzzy/fuzziness" is meant the agiographic appearance of the vessels in the retina, which are breaking down prior to drop out of the cell wall. As a result of this breakdown, the vessels are irregular in configuration and not able to contain the flouresceine dye used during a flouresceine angiagram (FA), which results in the vessels having a fuzzy appearance when visualized by FA.

"Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

By "inhibit" is meant a reduction in activity, level, or other measurable parameter relative to a reference (i.e., an untreated control). Such inhibition need not be complete, but can be by about 10%, 25%, 50%, 75% or more.

By "inhibitory nucleic acid" is meant a double-stranded RNA, siRNA, shRNA, or antisense RNA, or a portion thereof, or a mimetic thereof, that when administered to a mammalian cell results in a decrease (*e.g.*, by 10%, 25%, 50%, 75%, or even 90-100%) in the expression of a target gene. Typically, a nucleic acid inhibitor comprises at least a portion of a target nucleic acid molecule, or an ortholog thereof, or comprises at least a portion of the complementary strand of a target nucleic acid molecule. For example, an inhibitory nucleic acid molecule comprises at least a portion of any or all of the nucleic acids delineated herein.

By "isolated polynucleotide" is meant a nucleic acid (*e.g.*, a DNA) that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid

molecule of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. In addition, the term includes an RNA molecule that is transcribed from a DNA molecule, as well as a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By an "isolated polypeptide" is meant a polypeptide of the invention that has been separated from components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the invention. An isolated polypeptide of the invention may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding such a polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "marker" is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a retinal disease or disorder.

By "Norrin (NDP)" is meant a polypeptide or fragment thereof having at least about 85% amino acid identity to NCBI Accession No. NP_000257.1, as shown below, and having Wnt receptor-binding (e.g., by binding frizzled-4) activity that promotes retinal vascularization.

>gi|4557789|ref|NP_000257.1| norrin precursor [Homo sapiens]

MRKHVLAASFSMLSLLVIMGDTDSKTDSSFIMDSDPRRCMRHHYVDSISHPLYKCSSKMVLLARCEGHCSQASRS
EPLVSFSTVLKQPFRSSCHCCRPQTSKLKALRLRCSGGMRLTATYRYILSCHCEECNS

By "Norrin nucleic acid molecule" is meant a polynucleotide encoding a NDP polypeptide. An exemplary Norrin nucleic acid molecule is provided at NCBI Accession No. NM_000266.3, and is also shown below.

>gil223671892lreflNM_000266.3l Homo sapiens Norrie disease (pseudoglioma) (NDP), mRNA

AAGATGCTCCGTGGAAGGGAGCCGAGCGGTGGGCAGAGGCTGAGTCCCCGATAACGAGCGCCTCACATTT

CCGTGGCATTCCCATTTGCTAGTGCGCTGCTGCGGCCGCACGCCTGATTGATATATGACTGCAATGGCAC

TTTTCCATTTGACATTCTCTCTCTCTCTCTCCCTCTCTCTCTCTCCCTCTCTCTCTCCCTCTCTCTCTCT

CCCTGTGTCGCTTAAACAACAGTCCTAACTTTTGTGTGTTGCAAATATAAAAGGCAAGCCATGTGACAGA

GGGACAGAAGAACAAAAGCATTTGGAAGTAACAGGACCTCTTTCTAGCTCTCAGAAAAGTCTGAGAAGAA

AGGAGCCCTGCGTTCCCCTAAGCTGTGCAGCAGATACTGTGATGATGGATTGCAAGTGCAAAGAGTAAGA

CAAAACTCCAGCACATAAAGGACAATGACAACCAGAAAGCTTCAGCCCGATCCTGCCCTTTCCTTGAACG

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GAAGTTTTTCCTTACAACAATGAGAAAACATGTACTAGCTGCATCCTTTTCTATGCTCTCCCTGCTGGTG

ATAATGGGAGATACAGACAGTAAAACGGACAGCTCATTCATAATGGACTCGGACCCTCGACGCTGCATGA

GGCACCACTATGTGGATTCTATCAGTCACCCATTGTACAAGTGTAGCTCAAAGATGGTGCTCCTGGCCAG

GTGCGAGGGGCACTGCAGCCAGGCGTCACGCTCCGAGCCTTTGGTGTCGTTCAGCACTGTCCTCAAGCAA

CCCTTCCGTTCCTCCTGTCACTGCTGCCGGCCCCAGACTTCCAAGCTGAAGGCACTGCGGCTGCGATGCT

CAGGGGGCATGCGACTCACTGCCACCTACCGGTACATCCTCTCCTGTCACTGCGAGGAATGCAATTCCTG

AGGCCCGCTGCTGTGTGTGGCTTCTGGATGGGACAACTGTAGAGGCAGTTCGACCAGCCAGGGAAAGACT

GGCAAGAAAAGAGTTAAGGCAAAAAAGGATGCAACAATTCTCCCGGGACTCTGCATATTCTAGTAATAAA

GACTCTACATGCTTGTTGACAGAGAGAGATACTCTGGGAACTTCTTTGCAGTTCCCATCTCCTTTCTCTG

GTACAATTTCTTTTGGTTCATTTTCAGATTCAGGCATTTTCCCCCTTGGCTCTCAATGCTGTTTGGGTTT

CCAACAATTCAGCATTAGTGGGAAAAAGTGGGCCCTCATACACAAGCGTGTCAGGCTGTCAGTGTTTGGT

GCACGCTGGGGAAGAATTTACTTTGGAAAGTAGAAAAGCCCAGCTTTTCCTGGGACATCTTCTGTTATTG

TTGATGTTTTTTTTTACCTTGTCATTTTGGTCTAAGGTTGCCATTGCTGCTAAAGGTTACCGATTTCAAA

GTCCAGATACCAAGCATGTGGATATGTTTAGCTACGTTTACTCACAGCCAGCGAACTGACATTAAAATAA

CTAACAAACAGATTCTTTTATGTGATGCTGGAACTCTTGACAGCTATAATTATTATTCAGAAATGACTTT

TTGAAAGTAAAAGCAGCATAAAGAATTTGTCACAGGAAGGCTGTCTCAGATAAATTATGGTAAAATTTTG

TAAGGGAGCAGACTTTTAAAGACTTGCACAAATACGGATCCTGCACTGACTCTGGAAAAGGCATATATGT

ACTAGTGGCATGGAGAATGCACCATACTCATGCATGCAAATTAGACAACCAAGTATGAATCTATTTGTGG

GTGTGCTATAGCTTTAGCCGTGTCACGGGCATCATTCTCTAATATCCACTTGTCCATGTGAAACATGTTG

CCAAAATGGTGGCCTGGCTTGTCTTCTGAACGTTTGGTTCAAATGTGTTTTGGTCCTGGAGGCTCAAATT

TTGAGTTATTCCCACGTTTTGAAATAAAAAGAGTATATTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

AAAAAAAAAAAAAAAAAAAAAAAAAAAA

As used herein, "obtaining" as in "obtaining an agent" includes synthesizing, purchasing, or otherwise acquiring the agent.

The term "patient" or "subject" refers to an animal which is the object of treatment, observation, or experiment. By way of example only, a subject includes, but is not limited to, a mammal, including, but not limited to, a human or a non-human mammal, such as a non-human primate, bovine, equine, canine, ovine, or feline.

"Pharmaceutically acceptable" refers to approved or approvable by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, including humans.

"Pharmaceutically acceptable excipient, carrier or diluent" refers to an excipient, carrier or diluent that can be administered to a subject, together with an agent, and which does not destroy the pharmacological activity thereof and is nontoxic when administered in doses sufficient to deliver a therapeutic amount of the agent.

A "pharmaceutically acceptable salt" of a Wnt signaling activator or inhibitor recited herein is an acid or base salt that is generally considered in the art to be suitable for use in contact with the tissues of human beings or animals without excessive toxicity, irritation, allergic response, or other problem or complication. Such salts include mineral and organic acid salts of basic residues such as amines, as well as alkali or organic salts of acidic residues such as carboxylic acids. Specific pharmaceutical salts include, but are not limited to, salts of acids such as hydrochloric, phosphoric, hydrobromic, malic, glycolic, fumaric, sulfuric, sulfamic, sulfanilic, formic, toluenesulfonic, methanesulfonic, benzene sulfonic, ethane disulfonic, 2-hydroxyethylsulfonic, nitric, benzoic, 2-acetoxybenzoic, citric, tartaric, lactic, stearic, salicylic, glutamic, ascorbic, pamoic, succinic, fumaric, maleic, propionic, hydroxymaleic, hydroiodic, phenylacetic, alkanoic such as acetic, HOOC-$(CH_2)_n$-COOH where n is 0-4, and the like. Similarly, pharmaceutically acceptable cations include, but are not limited to sodium, potassium, calcium, aluminum, lithium and ammonium. Those of ordinary skill in the art will recognize further pharmaceutically acceptable salts for the Wnt signaling activators and inhibitors provided herein, including those listed by *Remington's Pharmaceutical Sciences,* 17th ed., Mack Publishing Company, Easton, PA, p. 1418 (1985). In general, a pharmaceutically acceptable acid or base salt can be synthesized from a parent compound that contains a basic or acidic moiety by any conventional chemical method.

Briefly, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in an appropriate solvent.

As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment," and the like, refer to reducing the probability of developing a disease or condition in a subject, who does not have, but is at risk of or susceptible to developing a disease or condition.

"Primer set" means a set of oligonucleotides that may be used, for example, for PCR. A primer set would consist of at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 80, 100, 200, 250, 300, 400, 500, 600, or more primers.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50, as well as all intervening decimal values between the aforementioned integers such as, for example, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, and 1.9. With respect to sub-ranges, "nested sub-ranges" that extend from either end point of the range are specifically contemplated. For example, a nested sub-range of an exemplary range of 1 to 50 may comprise 1 to 10, 1 to 20, 1 to 30, and 1 to 40 in one direction, or 50 to 40, 50 to 30, 50 to 20, and 50 to 10 in the other direction.

By "reduces" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

By "reference" is meant a standard or control condition.

A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the

reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

By "retinal disease" is meant any condition or disorder that damages or interferes with the normal function of the retina such as, for example, Diabetic Retinopathy, Proliferative Diabetic Retinopathy, Familial Exudative Vitreoretinopathy (FEVR), retinopathy of prematurity (ROP), Norries disease, Incognentia Pigmenti, branch retinal vein occlusion, central retinal vein occlusion, Coats disease, or Persistent Fetal-Vasculature syndrome.

By "siRNA" is meant a double stranded RNA. Optimally, an siRNA is 18, 19, 20, 21, 22, 23 or 24 nucleotides in length and has a 2 base overhang at its 3' end. These dsRNAs can be introduced to an individual cell or to a whole animal; for example, they may be introduced systemically via the bloodstream. Such siRNAs are used to downregulate mRNA levels or promoter activity.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the invention, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide of the invention.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (*e.g.*, a gene described herein), or portions thereof, under various

conditions of stringency. (See, *e.g.*, Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399; Kimmel, A. R. (1987) Methods Enzymol. 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, *e.g.*, formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30°C, more preferably of at least about 37°C, and most preferably of at least about 42°C. Varying additional parameters, such as hybridization time, the concentration of detergent, *e.g.*, sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred: embodiment, hybridization will occur at 30°C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42°C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25°C, more preferably of at least about 42°C, and even more preferably of at least about 68°C. In a preferred embodiment, wash steps will occur at 25°C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS.

Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel *et al.* (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

As used herein, the terms "treat," "treated," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition, or symptoms associated therewith be completely eliminated.

The term "therapeutic effect" refers to some extent of relief of one or more of the symptoms of a disorder or its associated pathology. "Therapeutically effective amount" as used herein refers to an amount of an agent which is effective, upon single or multiple dose

administration to the cell or subject, in prolonging the survivability of the patient with such a disorder, reducing one or more signs or symptoms of the disorder, preventing or delaying, and the like beyond that expected in the absence of such treatment. "Therapeutically effective amount" is intended to qualify the amount required to achieve a therapeutic effect. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the "therapeutically effective amount" (e.g., ED50) of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in a pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

Typically a therapeutically effective dosage should produce a serum concentration of compound of from about 0.1 ng/ml to about 50-100 µg/ml. The pharmaceutical compositions typically should provide a dosage of from about 0.001 mg to about 2000 mg of compound per kilogram of body weight per day. For example, dosages for systemic administration to a human patient can range from 1-10 µg/kg, 20-80 µg/kg, 5-50 µg/kg, 75-150 µg/kg, 100-500 µg/kg, 250-750 µg/kg, 500-1000 µg/kg, 1-10 mg/kg, 5-50 mg/kg, 25-75 mg/kg, 50-100 mg/kg, 100-250 mg/kg, 50-100 mg/kg, 250-500 mg/kg, 500-750 mg/kg, 750-1000 mg/kg, 1000-1500 mg/kg, 1500-2000 mg/kg, 5 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 500 mg/kg, 1000 mg/kg, 1500 mg/kg, or 2000 mg/kg. Pharmaceutical dosage unit forms are prepared to provide from about 1 mg to about 5000 mg, for example from about 100 to about 2500 mg of the compound or a combination of essential ingredients per dosage unit form.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a," "an," and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The phrase "combination therapy" embraces the administration of a Wnt signaling activator or inhibitor and a second therapeutic agent as part of a specific treatment regimen

intended to provide a beneficial effect from the co-action of these therapeutic agents. The beneficial effect of the combination includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. Administration of these therapeutic agents in combination typically is carried out over a defined time period (usually minutes, hours, days, or weeks depending upon the combination selected). "Combination therapy" generally is not intended to encompass the administration of two or more of these therapeutic agents as part of separate monotherapy regimens that incidentally and arbitrarily result in the combinations of the present invention. "Combination therapy" is intended to embrace administration of these therapeutic agents in a sequential manner, that is, wherein each therapeutic agent is administered at a different time, as well as administration of these therapeutic agents, or at least two of the therapeutic agents, in a substantially simultaneous manner. Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single capsule having a fixed ratio of each therapeutic agent or in multiple, single capsules for each of the therapeutic agents. For example, one combination of the present invention comprises a Wnt signaling activator or inhibitor and at least one additional therapeutic agent (e.g., an anti-viral agent, an immunosuppressive agent, an anti-inflammatory, and the like) at the same or different times or they can be formulated as a single, co-formulated pharmaceutical composition comprising the two compounds. As another example, a combination of the present invention (e.g., a Wnt signaling activator or inhibitor and at least one additional therapeutic agent) is formulated as separate pharmaceutical compositions that can be administered at the same or different time. Sequential or substantially simultaneous administration of each therapeutic agent can be effected by any appropriate route including, but not limited to, oral routes, intravenous routes, intramuscular routes, direct absorption through mucous membrane tissues (e.g., nasal, mouth, vaginal, and rectal), and ocular routes (e.g., intravitreal, intraocular, etc.). The therapeutic agents can be administered by the same route or by different routes. For example, one component of a particular combination may be administered by intravenous injection while the other component(s) of the combination may be administered orally. The components may be administered in any therapeutically effective sequence.

The phrase "combination" embraces groups of compounds or non-drug therapies useful as part of a combination therapy.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The

recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a fluorescein angiogram depicting a posterior capillary dropout phenotype as observed in the oxygen-induced retinopathy (OIR) mouse model.

Figure 2 is a montage of fluorescein angiograms depicting a retinal phenotype as observed in an OIR mouse, which highlights large areas of posterior capillary dropout in the retina.

Figure 3 is a montage of fluorescein angiograms depicting a retinal phenotype as observed in an OIR mouse in which Norrin/Wnt activity is blocked.

Figure 4 shows a fluorescein angiogram depicting posterior capillary sparing in the retina of an OIR mouse treated with intravitreal injection of Norrin.

Figure 5 shows a wide field fluorescein angiogram (Optos system) of a person with Familial Exudative Vitreoretinopathy (FEVR), who has had previous laser treatment of avascular retina (far right of the figure). Healthy vessels are observed in the back of the retina in the vicinity of the optic disc. Adjacent to the treated area, there is an area of fuzziness corresponding to damaged vessels showing leakage of dye from damaged endothelial cells (arrow). These changes precede frank capillary loss, which is what drives reactivation of the acute process of FEVR, retinal destruction, and vision loss.

Figure 6 depicts a higher magnification view of the area of capillary damage shown in FIG. 5. Shown are small areas of microanyeurisms and endothelial cell damage as manifested by dye leakage. Left alone this area will result in complete capillary loss.

Figure 7 shows a low magnification wide field fluorescein angiogram (Optos) of a retina of a patient with diabetes prior to the onset of macular diabetic retinopathy (e.g., either background diabetic retinopathy or macular diabetic retinopathy), which reveals numerous small white dots that correspond to microaneurisms surrounding areas of capillary drop out prior to

any change in the macula area. These microaneurism-associated areas are believed to give rise to macular diabetic retinopathy, and according to any exemplary embodiment of the invention, these areas represent target areas for treatment with an activator of Wnt signaling (e.g., Norrin).

Figure 8 shows a high magnification view of the wide field fluorescein angiogram shown in FIG. 7.

Figure 9 shows a regular fluorescein angiogram depicting a normal macula.

Figure 10 shows a wide field fluorescein angiogram depicting a normal macula.

DETAILED DESCRIPTION OF THE INVENTION

The present invention features compositions and methods that are useful in treating retinal diseases. In particular, the invention provides compositions comprising Wnt signaling enhancing compounds for the treatment of retinal diseases such as, for example, familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), Norries disease, branch retinal vein occlusion, and/or central retinal vein occlusion.

The invention is based, at least in part, on the discovery that agents that enhance Wnt signaling (e.g., Norrin) have the ability to treat and/or prevent capillary dropout, thereby treating and/or preventing many forms of retinal disease (e.g., FEVR, diabetic retinopathy, ROP, branch retinal vein occlusion, central retinal vein occlusion, and the like). Additionally, the invention is also based, at least in part, on the discovery that antecedent lesions represent an early prognostic marker for the above-mentioned retinal diseases that may allow treatment prior to a patient's development of symptoms of vision loss. The invention features compositions and kits containing Wnt signaling enhancing compounds. The invention also features methods for using these novel therapeutic molecules to treat a subject having, or at risk of having, a retinal disease such as, for example, familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), Norries disease, branch retinal vein, or central retinal vein occlusion.

Retinal Vascularization

Proper vascular modeling in the retina is essential for ocular development and visual acuity. Abnormal vessel growth during development or in adulthood produces several relatively common diseases such as retinopathy of prematurity, diabetic retinopathy, and age-

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related macular degeneration. Normal retinal development occurs through vessels forming at the optic nerve head and spreading over the retina to form a dense network. Connolly, S E, et al, Microvasc Res, 1988; 36:275-290; Provis, J M, Prog Retin Eye Res, 2001; 20:799-821; Fruttiger, M, Invest Ophthalmol Vis Sci, 2002; 43:522-527. Development proceeds through formation of primary vessels along the surface of the developing retina from which divergent vessels begin to extend into the capillary beds that form the outer and inner plexiform layers of the retina. Connelly, 1988; Provis, 2001, Fruttiger, 2002. Vascular development is mediated by a series of growth factors that direct formation and extension of new vessels. Retinal development is unique in the concentration and types of signaling mediators employed to promote angiogenic sprouting from the primary vascular network and the formation of the final capillary architecture. Ohlmann, A, et al, J Neurosci, 2005; 25:1701-1710.

Wnt Signaling Pathway

There are three described Wnt pathways, one of which, the canonical ß-catenin pathway, plays a role in gene regulation and cell proliferation of both vascular and neural tissue development. The other two pathways, the planar cell polarity and the calcium Ca2+ plush pathways, participate in cell migration and cytoskeletal structure and cell adhesion, as well as protein kinase C activity.

In the canonical Wnt pathway, Wnt proteins (i.e., ligands) bind to cell-surface receptors of the Frizzled family, which causes the receptors to activate Dishevelled (DSH) family proteins. Receptors of the Frizzled family may be associated with co-receptors such as LRP5 and LRP6. DSH activation ultimately results in a change in the amount of β-catenin that reaches the nucleus. DSH is a key component of a membrane-associated Wnt receptor complex, which, when activated by Wnt binding, inhibits a second complex of proteins that includes axin, GSK-3, and the protein APC. The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signaling molecule. However, once the "β-catenin destruction complex" is inhibited, a stabilized pool of cytoplasmic β-catenin is created, which allows some β-catenin to be able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression patters (see, e.g., R. Baron and M. Kneissel "WNT signaling in bone homeostasis and disease: from human mutations to treatments" Nature Medicine 19(2):179-192).

Wnt signaling has been well described in Norrie disease and to a large extent in FEVR. It has also been shown in an animal model that supplementation with Norrin (the disease gene product of Norrie disease) can avoid the phenotypic presentation of Norrie disease–type changes in these animal models by supplementing Norrin to drive the Wnt signaling pathway.

One factor hypothesized to be involved in formation of primary retinal vasculature and retinal capillaries is the protein Norrin. Norrin (NDP), is a 131 amino acid long protein that is secreted into the extracellular space. Meitinger, T, et al, Nat Genet, 1993; 5:376-380; Berger, W, et al, Hum Mol Genet, 1996; 5:51-59. Two primary domains define the general Norrin protein structure: a signal peptide directs localization of the molecule; and a cysteine-knot motif provides the tertiary confirmation required for receptor binding and activation of signal transduction.

The importance of the cysteine knot-motif is highlighted by computer modeling that demonstrates the requirement of disulfide bonds between the cysteine residues in forming the structural confirmation of Norrin. Mutation(s) of the cysteine residues reduces the affinity of Norrin for its receptor and prevents activation of subsequent signaling pathways. Mutations in these residues also result in severe retinal dysgenesis and Norrie disease. However, mutations in regions other than the cysteine knot-motif produce incomplete protein folding and result in familial exudative vitreoretinopathy (FEVR) and related vitreoretinopathies (Retinopathy of Prematurity, persistent fetal vasculature).

Norrin is a ligand for the Frizzled receptor subtype 4 (Fz4). Norrin binds Fz4 with nanomolar affinity and stimulates a Wnt receptor:β-catenin signal transduction pathway that regulates retinal development and is necessary for regression of hyaloid vessels in the eye. Xu, Q, et al, Cell, 2004; 116:883-895; Clevers, H, Curr Biol, 2004; 14:R436-437; Nichrs, C, Dev Cell, 2004; 6:453-454. Norrin interaction with Fz4 is dependent on the cell surface receptor LRP5. Xu, 2004. Frizzled receptors are coupled to the β-catenin canonical signaling pathway that functions by the activation of Wnt target genes. Wnt protein binding to Frizzled and LRP5 inactivates glycogen synthase kinase (GSK) 3β and Axin. The inactivation of these proteins stabilizes β-catenin, which subsequently accumulates in the cell nucleus and activates the transduction of target genes that are crucial in the G1-S-phase transition, such as cyclin D1 or c-Myc. Willert K, and Nusse R, Curr Opin Genet Dev, 1998; 8:95-102. These

pathways promote stimulation and proliferation of retinal stem cells. Inoue, T, et al, Stem Cells, 2006; 24:95-104.

Norrin is encoded by the NDP gene present on chromosome X at position 11.4. The importance of this gene product is highlighted by observations that inactivating mutations lead to Norrie disease, which is characterized by ocular and cochlear vascular defects. Rhem, H L, et al, J Neurosci, 2002; 22:4286-4292; Black, G C, et al, Hum Mol Genet, 1999; 8:2031-2035. Silencing of the NDP gene produces incomplete regression of the primary hyaloid system and abnormal retinal maturation.

Observations that abnormalities in the Fz4 and LRP5/LRP6 receptors that result in the phenotypically similar condition, FEVR, underscore the importance of Norrin signaling. Robitaille, J, et al, Nature Genet, 2002; 32:326-330; Kondo, H, et al, Br J Opthalmol, 2003; 87:1291-1295; Toomes, C, et al, Am J Hum Genet, 2004; 74:721-730. The close association between the phenotypes produced by Norrin mutations and mutations in the Fz4 and LRP5 receptors bolsters the hypothesis that these molecules form a functional signaling group. Planutis, K, et al, BMC Cell Biology, 2007; 8:12.

Wnt Signaling Enhancing Compounds

In general, the invention features Wnt signaling enhancing compounds that are useful in treating or preventing retinal disease. The Wnt signaling enhancing compounds can be any agent (e.g., a small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide) that enhances Wnt signaling. In embodiments, Wnt signaling enhancing compounds comprise Frizzled (Fz) receptors, Fz co-receptors such as LRP5 and LRP6, axin, GSK-3, APC, DSH, or any other effectors of Wnt signaling downstream of Norrin. In embodiments, the Wnt signaling enhancing compounds are Fz4 specific activators. In an embodiment, the Wnt signaling enhancing compound comprises a small molecule. In preferred embodiments, the Wnt signaling enhancing compound is a polypeptide or polypeptide fragment. In related embodiments, the Wnt signaling enhancing compound is a Norrin polypeptide, or active fragment thereof, that is a Wnt signaling activator. In related embodiments, the Wnt signaling enhancing compound is 2-amino-4-[3,4-(methylenedioxy)benzyl-amino]-6-(3-methoxyphenyl)pyrimidine.

In embodiments, the Wnt signaling enhancing compounds comprises a Norrin polypeptide with the following amino acid sequence:

MRKHVLAASFSMLSLLVIMGDTDSKTDSSFIMDSDPRRCMRHHYVDSISHPLYKCSSKM VLLARCEGHCSQASRSEPLVSFSTVLKQPFRSSCHCCRPQTSKLKALRLRCSGGMRLTAT YRYILSCHCEECNS. In other embodiments, the Wnt signaling enhancing compounds comprise and active fragment of the Norrin polypeptide. Activity of a Norrin polypeptide, or an active fragment thereof, may be assessed by any of a variety of Wnt signaling assays known to one of skill in the art (see, e.g., US Publication No. 2010/0239499, which is hereby incorporated by reference in its entirety for all purposes).

In any of the above aspects and embodiments, the Wnt signaling enhancing compound(s) further contains a targeting moiety. In embodiments, the targeting moiety facilitates delivery of the Wnt signaling enhancing compound(s) to the Wnt receptor (e.g., Fz4). In related embodiments, the targeting moiety contains any of a number of cell penetrating domains known to one of skill in the art such as, e.g., trans-activating transcriptional activator (Tat) from HIV-1 (see Wadia *et al.*, *Nat. Med.* 10:310-315 (2004); and Kameyama *et al.*, *Bioconjugate Chem.* 17:597–602 (2006)) and Pep-1 (see Morris *et al.*, *Nat. Biotechnol.* 19:1173–1176 (2001)).

In any of the above aspects and embodiments, the Wnt signaling enhancing compound(s) further contains a detectable moiety. Detectable moieties are well known in the art and can be detected by spectroscopic, photochemical, biochemical, immunochemical, physical, or chemical means. Exemplary moieties include, but are not limited to, enzymes, fluorescent molecules, particle labels, electron-dense reagents, radiolabels, biotin, digoxigenin, or a hapten or a protein that has been made detectable.

The Wnt signaling enhancing compound(s) can be covalently or non-covalently linked to a moiety (e.g., targeting moiety and/or detectable moiety). In embodiments, the Wnt signaling enhancing compound(s) are covalently linked to the moiety. In related embodiments, the covalent linkage of the moiety is N-terminal to the polypeptide fragment. In related embodiments, the covalent linkage of the moiety is C-terminal to the peptide fragment.

The invention further embraces variants and equivalents which are substantially homologous to the Wnt signaling enhancing compound(s) described herein. These can contain, for example, conservative substitution mutations, i.e., the substitution of one or more amino acids by similar amino acids. For example, conservative substitution refers to the substitution of an amino acid with another within the same general class such as, for example, one acidic amino acid with another acidic amino acid, one basic amino acid with another

basic amino acid, or one neutral amino acid by another neutral amino acid. What is intended by a conservative amino acid substitution is well known in the art.

The invention also provides isolated polypeptides of the activators of the invention, as well as isolated polynucleotides encoding the polypeptides. In addition, the invention further provides expression vectors comprising the isolated polynucleotides, as well as host cells containing the expression vectors.

The term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequences for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequences. The polynucleotides of the invention can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand.

The present invention further relates to variants of the polynucleotides, for example, fragments, analogs, and derivatives. The variant of the polynucleotide can be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide. In certain embodiments, the polynucleotide can have a coding sequence which is a naturally occurring allelic variant of the coding sequence of the disclosed polypeptides. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence that have, for example, a substitution, deletion, or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

In embodiments, the polynucleotides can comprise the coding sequence for the mature polypeptide fused in the same reading frame to a polynucleotide which aids, for example, in expression and secretion of a polypeptide from a host cell (e.g., a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell). The polypeptide having a leader sequence is a preprotein and can have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides can also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

In embodiments, the polynucleotides can comprise the coding sequence for the mature polypeptide fused in the same reading frame to a marker sequence that allows, for example, for purification of the encoded polypeptide. For example, the marker sequence can be a

hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or the marker sequence can be a hemagglutinin (HA) tag derived from the influenza hemagglutinin protein when a mammalian host (e.g., COS-7 cells) is used. Additional tags include, but are not limited to, Calmodulin tags, FLAG tags, Myc tags, S tags, SBP tags, Softag 1, Softag 3, V5 tag, Xpress tag, Isopeptag, SpyTag, Biotin Carboxyl Carrier Protein (BCCP) tags, GST tags, fluorescent protein tags (e.g., green fluorescent protein tags), maltose binding protein tags, Nus tags, Strep-tag, thioredoxin tag, TC tag, Ty tag, and the like.

In embodiments, the present invention provides isolated nucleic acid molecules having a nucleotide sequence at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, or at least 96%, 97%, 98% or 99% identical to a polynucleotide encoding a polypeptide comprising Wnt signaling enhancing compound(s) of the present invention.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence can include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence can be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence can be inserted into the reference sequence. These mutations of the reference sequence can occur at the amino- or carboxy-terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 80% identical, at least 85% identical, at least 90% identical, and in some embodiments, at least 95%, 96%, 97%, 98%, or 99% identical to a reference sequence can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482 489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other

sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some embodiments, the polynucleotide variants contain alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In some embodiments, nucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. Polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

The polypeptides of the present invention can be recombinant polypeptides, natural polypeptides, or synthetic polypeptides comprising a Wnt signaling enhancing compound(s) as described herein. It will be recognized in the art that some amino acid sequences of the invention can be varied without significant effect of the structure or function of the protein. Thus, the invention further includes variations of the polypeptides which show substantial activity or which include regions of the Wnt signaling enhancing compound(s) of the present invention. Such mutants include deletions, insertions, inversions, repeats, and type substitutions.

The polypeptides and analogs can be further modified to contain additional chemical moieties not normally part of the protein. Those derivatized moieties can improve the solubility, the biological half-life, absorption of the protein, or binding affinity. The moieties can also reduce or eliminate any desirable side effects of the proteins and the like. An overview for those moieties can be found in Remington's Pharmaceutical Sciences, 20th ed., Mack Publishing Co., Easton, PA (2000).

The isolated polypeptides described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthetic methods to constructing a DNA sequence encoding isolated polypeptide sequences and expressing those sequences in a suitable transformed host. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating or synthesizing a DNA sequence encoding a wild-type protein of interest. Optionally, the sequence can be mutagenized by site-specific mutagenesis

to provide functional analogs thereof. *See, e.g.* Zoeller et al., *Proc. Nat'l. Acad. Sci.* USA 81:5662-5066 (1984) and U.S. Pat. No. 4,588,585.

In embodiments, a DNA sequence encoding a polypeptide of interest would be constructed by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize an isolated polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled (e.g., by synthesis, site-directed mutagenesis, or another method), the polynucleotide sequences encoding a particular isolated polypeptide of interest will be inserted into an expression vector and optionally operatively linked to an expression control sequence appropriate for expression of the protein in a desired host. Proper assembly can be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable host. As well known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene can be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host.

Recombinant expression vectors are used to amplify and express DNA encoding the Wnt signaling enhancing compound(s) . Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding a Wnt signaling enhancing compound(s) or a bioequivalent analog operatively linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements can include an operator sequence to control transcription. The ability to replicate in a host, usually conferred

by an origin of replication, and a selection gene to facilitate recognition of transformants can additionally be incorporated. DNA regions are operatively linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operatively linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operatively linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operatively linked to a coding sequence if it is positioned so as to permit translation. Generally, operatively linked means contiguous, and in the case of secretory leaders, means contiguous and in reading frame. Structural elements intended for use in yeast expression systems include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it can include an N-terminal methionine residue. This residue can optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *Escherichia coli*, including pCR 1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as M13 and filamentous single-stranded DNA phages.

Suitable host cells for expression of a polypeptide include prokaryotes, yeast, insect or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *bacilli*. Higher eukaryotic cells include established cell lines of mammalian origin. Cell-free translation systems could also be employed. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are well known in the art (see Pouwels *et al.*, *Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y., 1985).

Various mammalian or insect cell culture systems are also advantageously employed to express recombinant protein. Expression of recombinant proteins in mammalian cells can be performed because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell* 23:175, 1981), and other cell

lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors can comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

The proteins produced by a transformed host can be purified according to any suitable method. Such standard methods include chromatography (e.g., ion exchange, affinity and sizing column chromatography, and the like), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence, glutathione-S-transferase, and the like can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance and x-ray crystallography.

For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a cancer stem cell protein-Fc composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant protein produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid

chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Methods of Treatment

The invention includes methods for treating or preventing retinal disease with the Wnt signaling enhancing compound(s) described herein.

In aspects, the invention provides methods for enhancing Wnt signaling in a cell. In embodiments, the methods involve enhancing Wnt signaling with a Norrin polypeptide. In related embodiments, the methods involve contacting the cell with a Norrin polypeptide, or a fragment thereof. In related embodiments, the methods involve contacting the cell with 2-amino-4-[3,4-(methylenedioxy)benzyl-amino]-6-(3-methoxyphenyl)pyrimidine.

In embodiments, the cell is in the retina of a subject. In related embodiments, contacting occurs by therapeutic administration of the inhibitor to the retina of the subject in the form of a pharmaceutical composition.

In any of the above aspects and embodiments, the methods may further involve contacting the retinal cell with, or administering to the retina of a subject, an anti-viral agent, an immunosuppressive agent, or an anti-inflammatory.

In any of the above aspects and embodiments, the subject is a mammal (e.g., human) or the cell is from a mammal (e.g., human).

Methods for evaluating the therapeutic efficacy of the methods of the invention are standard in the art. For example, efficacy of treatment can be evaluated by assessing retinal vascularization (e.g., by regular or wide field FA), patient symptoms, visual acuity, and the like.

Pharmaceutical Compositions/Methods of Delivery

The present invention is also directed to pharmaceutical compositions comprising an effective amount of one or more compounds according to the present invention (including a pharmaceutically acceptable salt, thereof), optionally in combination with a pharmaceutically acceptable carrier, excipient or additive.

A "pharmaceutically acceptable derivative or prodrug" means any pharmaceutically acceptable salt, ester, salt of an ester, or other derivative of a compound of this invention which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of this invention. Particularly favored derivatives and prodrugs are those that increase the bioavailability of the compounds of this invention when such compounds are administered to a mammal (e.g., by allowing an orally or ocularly administered compound to be more readily absorbed into the blood) or which enhance delivery of the parent compound to a biological compartment (e.g., the retina) relative to the parent species.

While the Wnt signaling enhancing compounds of the invention can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more compounds of the invention or other agents. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

The Wnt signaling enhancing compounds of the present invention may be administered orally, parenterally, by inhalation spray, rectally, vaginally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intramuscular, intrasternal, infusion techniques, intraperitoneally, eye or ocular, intravitreal, intrabuccal, transdermal, intranasal, into the brain, including intracranial and intradural, into the joints, including ankles, knees, hips, shoulders, elbows, wrists, directly into tumors, and the like, and in suppository form.

The pharmaceutically active compounds of this invention can be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, including humans and other mammals.

Modifications of the active compound can affect the solubility, bioavailability and rate of metabolism of the active species, thus providing control over the delivery of the active species. Further, the modifications can affect the anti-angiogenesis activity of the compound, in some cases increasing the activity over the parent compound. This can easily be assessed by preparing the derivative and testing its activity according to known methods well within the routine practitioner's skill in the art.

Pharmaceutical compositions based upon these chemical compounds comprise the above-described Wnt signaling enhancing compounds in a therapeutically effective amount for treating diseases and conditions which have been described herein, optionally in combination with a pharmaceutically acceptable additive, carrier and/or excipient. One of ordinary skill in the art will recognize that a therapeutically effective amount of one of more compounds according to the present invention will vary with the infection or condition to be treated, its severity, the treatment regimen to be employed, the pharmacokinetics of the agent used, as well as the patient (animal or human) treated.

To prepare the pharmaceutical compositions according to the present invention, a therapeutically effective amount of one or more of the compounds according to the present invention is preferably intimately admixed with a pharmaceutically acceptable carrier according to conventional pharmaceutical compounding techniques to produce a dose. A carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., ocular, oral, topical or parenteral, including gels, creams ointments, lotions and time released implantable preparations, among numerous others. In preparing pharmaceutical compositions in oral dosage form, any of the usual pharmaceutical media may be used. Thus, for liquid oral preparations such as suspensions, elixirs and solutions, suitable carriers and additives including water, glycols, oils, alcohols, flavouring agents, preservatives, colouring agents and the like may be used. For solid oral preparations such as powders, tablets, capsules, and for solid preparations such as suppositories, suitable carriers and additives including starches, sugar carriers, such as dextrose, mannitol, lactose and related carriers, diluents, granulating agents, lubricants, binders, disintegrating agents and the like may be used. If desired, the tablets or capsules may be enteric-coated or sustained release by standard techniques.

The active compound is included in the pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount for the desired indication, without causing serious toxic effects in the patient treated.

Oral compositions will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound or its prodrug derivative can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically

compatible binding agents, and/or adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a dispersing agent such as alginic acid or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material-of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or enteric agents.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil emulsion and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets optionally may be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Methods of formulating such slow or controlled release compositions of pharmaceutically active ingredients, are known in the art and described in several issued US Patents, some of which include, but are not limited to, US Patent Nos. 3,870,790; 4,226,859; 4,369,172; 4,842,866 and 5,705,190, the disclosures of which are incorporated herein by reference in their entireties. Coatings can be used for delivery of compounds to the intestine (see, e.g., U.S. Patent Nos. 6,638,534, 5,541,171, 5,217,720, and 6,569,457, and references cited therein).

The active compound or pharmaceutically acceptable salt thereof may also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose or fructose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

Solutions or suspensions used for ocular, parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

A skilled artisan will recognize that in addition to tablets, other dosage forms can be formulated to provide slow or controlled release of the active ingredient. Such dosage forms include, but are not limited to, capsules, granulations and gel-caps.

Liposomal suspensions may also be pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art. For example, liposomal formulations may be prepared by dissolving appropriate lipid(s) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound are then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension. Other methods of preparation well known by those of ordinary skill may also be used in this aspect of the present invention.

The formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of

bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations and compositions suitable for topical administration in the mouth include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

Formulations suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising the ingredient to be administered in a pharmaceutical acceptable carrier. A preferred topical delivery system is a transdermal patch containing the ingredient to be administered.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of 20 to 500 microns which is administered in the manner in which snuff is administered, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations, wherein the carrier is a liquid, for administration, as for example, a nasal spray or as nasal drops, include aqueous or oily solutions of the active ingredient.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. If administered intravenously, preferred carriers include, for example, physiological saline or phosphate buffered saline (PBS).

For parenteral formulations, the carrier will usually comprise sterile water or aqueous sodium chloride solution, though other ingredients including those which aid dispersion may

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be included. Of course, where sterile water is to be used and maintained as sterile, the compositions and carriers must also be sterilized. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Administration of the active compound may range from continuous (intravenous drip) to several oral administrations per day (for example, Q.I.D.) and may include oral, topical, eye or ocular, parenteral, intramuscular, intravenous, sub-cutaneous, transdermal (which may include a penetration enhancement agent), buccal and suppository administration, among other routes of administration, including through an eye or ocular route.

Application of the subject therapeutics may be local, so as to be administered at the site of interest. Various techniques can be used for providing the subject compositions at the site of interest, such as injection, use of catheters, trocars, projectiles, pluronic gel, stents, sustained drug release polymers or other device which provides for internal access. Where an organ or tissue is accessible because of removal from the patient, such organ or tissue may be bathed in a medium containing the subject compositions, the subject compositions may be painted onto the organ, or may be applied in any convenient way.

The Wnt signaling enhancing compounds may be administered through a device suitable for the controlled and sustained release of a composition effective in obtaining a desired local or systemic physiological or pharmacological effect. The method includes positioning the sustained released drug delivery system at an area wherein release of the agent is desired and allowing the agent to pass through the device to the desired area of treatment.

More specifically, the Wnt signaling enhancing compound is administered through an ocular device suitable for direct implantation into the vitreous of the eye. Such devices of the present invention are surprisingly found to provide sustained controlled release of various compositions to treat the eye without risk of detrimental local and systemic side effects. An object of the present ocular method of delivery is to maximize the amount of drug contained in an intraocular device while minimizing its size in order to prolong the duration of the implant. See, e.g., U.S. Patents 5,378,475; 5,773,019; 6,001,386; 6,217,895, 6,375,972, and 6,756,058 and U.S. Publications 20050096290 and 200501269448.

Other methods of delivery of Wnt signaling enhancing compounds include: an ocular delivery system that could be applied to an intra-ocular lens to prevent inflammation or posterior capsular opacification, an ocular delivery system that could be inserted directly into the vitreous, under the retina, or onto the sclera, and wherein inserting can be achieved by injecting the system or surgically implanting the system, a sustained release drug delivery system, and a method for providing controlled and sustained administration of an agent effective in obtaining a desired local or systemic physiological or pharmacological effect comprising surgically implanting a sustained release drug delivery system at a desired location.

Examples include, but are not limited to the following: a sustained release drug delivery system comprising an inner reservoir comprising an effective amount of an agent effective in obtaining a desired local or systemic physiological or pharmacological effect, an inner tube impermeable to the passage of said agent, said inner tube having first and second ends and covering at least a portion of said inner reservoir, said inner tube sized and formed of a material so that said inner tube is capable of supporting its own weight, an impermeable member positioned at said inner tube first end, said impermeable member preventing passage of said agent out of said reservoir through said inner tube first end, and a permeable member positioned at said inner tube second end, said permeable member allowing diffusion of said agent out of said reservoir through said inner tube second end; a method for administering a compound of the invention to a segment of an eye, the method comprising the step of implanting a sustained release device to deliver the compound of the invention to the vitreous of the eye or an implantable, sustained release device for administering a compound of the invention to a segment of an eye; a sustained release drug delivery device comprising: a) a drug core comprising a therapeutically effective amount of at least one first agent effective in

obtaining a diagnostic effect or effective in obtaining a desired local or systemic physiological or pharmacological effect; b) at least one unitary cup essentially impermeable to the passage of said agent that surrounds and defines an internal compartment to accept said drug core, said unitary cup comprising an open top end with at least one recessed groove around at least some portion of said open top end of said unitary cup; c) a permeable plug which is permeable to the passage of said agent, said permeable plug is positioned at said open top end of said unitary cup wherein said groove interacts with said permeable plug holding it in position and closing said open top end, said permeable plug allowing passage of said agent out of said drug core, through said permeable plug, and out said open top end of said unitary cup; and d) at least one second agent effective in obtaining a diagnostic effect or effective in obtaining a desired local or systemic physiological or pharmacological effect; or a sustained release drug delivery device comprising: an inner core comprising an effective amount of an agent having a desired solubility and a polymer coating layer, the polymer layer being permeable to the agent, wherein the polymer coating layer completely covers the inner core.

The Wnt signaling enhancing compounds may be administered as microspheres. For example, Norrin may be purchased from R&D Systems, Minneapolis, Minn., or cloned, expressed and purified is loaded into biodegradable microspheres substantially as described by Jiang, C, et al, Mol Vis, 2007; 13:1783-92 using the spontaneous emulsification technique of Fu, K, et al, J Pharm Sci, 2003; 92:1582-91. Microspheres are synthesized and loaded by dissolving 200 mg of 50:50 PLGA (DURECT Corp., Birmingham, Ala.) in 5 ml of 4:1 volume ratio trifluoroethanol:dichloromethane supplemented with 8 mg magnesium hydroxide to minimize protein aggregation during encapsulation. 10 μg Norrin may be reconstituted in 300 μl 7 mg bovine serum albumin (BSA) and 100 mg docusate sodium (Sigma-Aldrich, St. Louis, Mo.) dissolved in 3 ml PBS. The solution may be vortexed and poured into 200 ml of 1% (w/v) polyvinyl alcohol (PVA, 88% hydrolyzed) with gentle stirring. Microspheres may be hardened by stirring for three hours, collected by centrifugation, and washed three times to remove residual PVA. If the microspheres are not to be immediately injected they are rapidly frozen in liquid nitrogen, lyophilized for 72 h, and stored in a dessicator at −20° C. Norrin containing microspheres exhibit average diameters of 8 μm as determined by a particle size.

The Wnt signaling enhancing compounds may also be administered by intravitreal injection. For example, Norrin in solution, may be packaged into microspheres as described above, or expressed in cells, or in purified form in solution may be exposed to the retina by intravitreal injection substantially as described by Jiang, 2007. Intravitreal injection may be performed under general anesthesia using an ophthalmic operating microscope (Möller-Wedel GmbH, Wedel, Germany) using beveled glass micro-needles with an outer diameter of approximately 100 μm. Microsphere suspensions are prepared in PBS at 2 and 10% (w/v) and briefly vortexed immediately before injection to ensure a uniform dispersion. A 30-gauge hypodermic needle may be used to perforate the sclera 1.5 mm behind the limbus. Five μl of test sample is optionally injected by way of this passage into the vitreous using a 50 μl Hamilton Syringe (Hamilton Co, Reno, Nev.). To ensure adequate delivery and prevent shock the needle is held in place for one min after the injection is completed and subsequently withdrawn slowly. In addition, paracentesis may be simultaneously performed to relieve pressure and thereby prevent reflux.

The Wnt signaling enhancing compounds may also be administered by delivery to the retina by a controlled release delivery system. An implantable controlled release delivery system is described in U.S. Patent Application Publication 2005/0281861 A1 which is incorporated herein by reference for its entire disclosure, figures, examples, and methods. Norrin is packaged into such as system at 100 μg per final formulated capsule. For example, a Norrin containing drug delivery systems may be placed in the eye using forceps or a trocar after making a 2-3 mm incision in the sclera. Alternatively, no incision may be made and the system placed in an eye by inserting a trocar or other delivery device directly through the eye. The removal of the device after the placement of the system in the eye can result in a self-sealing opening. One example of a device that is used to insert the implants into an eye is disclosed in U.S. Patent Application Publication No. 2004/0054374 which is incorporated herein by reference. The location of the system may influence the concentration gradients of therapeutic component or drug surrounding the element, and thus influence the release rates (e.g., an element placed closer to the edge of the vitreous may result in a slower release rate). Thus, it is preferred if the system is placed near the retinal surface or in the posterior portion of the vitreous.

The above methods are particularly suitable for treating ocular conditions such as familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), and/or Norries disease.

The Wnt signaling enhancing compound may be utilized in combination with at least one known other therapeutic agent, or a pharmaceutically acceptable salt of said agent. Examples of known therapeutic agents which can be used for combination therapy include, but are not limited to, corticosteroids (e.g., cortisone, prodnisone, dexamethasone), non-steroidal anti-inflammatory drugs (NSAIDS) (e.g., ibuprofen, celecoxib, aspirin, indomethicin, naproxen), alkylating agents such as busulfan, cis-platin, mitomycin C, and carboplatin; antimitotic agents such as colchicine, vinblastine, paclitaxel, and docetaxel; topo I inhibitors such as camptothecin and topotecan; topo II inhibitors such as doxorubicin and etoposide; and/or RNA/DNA antimetabolites such as 5-azacytidine, 5-fluorouracil and methotrexate; DNA antimetabolites such as 5-fluoro-2′-deoxy-uridine, ara-C, hydroxyurea and thioguanine; antibodies such as Herceptin® and Rituxan®.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

In certain pharmaceutical dosage forms, the pro-drug form of the compounds may be preferred. One of ordinary skill in the art will recognize how to readily modify the present compounds to pro-drug forms to facilitate delivery of active compounds to a targeted site within the host organism or patient. The routine practitioner also will take advantage of favorable pharmacokinetic parameters of the pro-drug forms, where applicable, in delivering the present compounds to a targeted site within the host organism or patient to maximize the intended effect of the compound.

Preferred prodrugs include derivatives where a group which enhances aqueous solubility or active transport through the gut membrane is appended to the structure of formulae described herein. See, e.g., Alexander, J. et al. *Journal of Medicinal Chemistry* **1988**, *31*, 318-322; Bundgaard, H. *Design of Prodrugs*; Elsevier: Amsterdam, 1985; pp 1-92; Bundgaard, H.; Nielsen, N. M. *Journal of Medicinal Chemistry* **1987**, *30*, 451-454; Bundgaard, H. *A Textbook of Drug Design and Development*; Harwood Academic Publ.:

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Switzerland, 1991; pp 113-191; Digenis, G. A. et al. *Handbook of Experimental Pharmacology* **1975**, *28*, 86-112; Friis, G. J.; Bundgaard, H. *A Textbook of Drug Design and Development*; 2 ed.; Overseas Publ.: Amsterdam, 1996; pp 351-385; Pitman, I. H. *Medicinal Research Reviews* **1981**, *1*, 189-214. The prodrug forms may be active themselves, or may be those such that when metabolized after administration provide the active therapeutic agent *in vivo*.

Pharmaceutically acceptable salt forms may be the preferred chemical form of compounds according to the present invention for inclusion in pharmaceutical compositions according to the present invention.

Certain of the compounds, in pharmaceutical dosage form, may be used as agents for preventing a disease or condition from manifesting itself. In certain pharmaceutical dosage forms, the pro-drug form of the compounds according to the present invention may be preferred. In particular, prodrug forms which rely on C_1 to C_{20} ester groups or amide groups (preferably a hydroxyl, free amine or substituted nitrogen group) which may be transformed into, for example, an amide or other group may be particularly useful in this context.

The present compounds or their derivatives, including prodrug forms of these agents, can be provided in the form of pharmaceutically acceptable salts. As used herein, the term pharmaceutically acceptable salts or complexes refers to appropriate salts or complexes of the active compounds according to the present invention which retain the desired biological activity of the parent compound and exhibit limited toxicological effects to normal cells. Nonlimiting examples of such salts are (a) acid addition salts formed with inorganic acids (for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like), and salts formed with organic acids such as acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, and polyglutamic acid, among others; (b) base addition salts formed with metal cations such as zinc, calcium, sodium, potassium, and the like, among numerous others.

The compounds herein are commercially available or can be synthesized. As can be appreciated by the skilled artisan, further methods of synthesizing the compounds of the formulae herein will be evident to those of ordinary skill in the art. Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies

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(protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, *Comprehensive Organic Transformations*, 2nd. Ed., Wiley-VCH Publishers (1999); T.W. Greene and P.G.M. Wuts, *Protective Groups in Organic Synthesis*, 3rd. Ed., John Wiley and Sons (1999); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1999); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995), and subsequent editions thereof.

The compounds of this invention may contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of these compounds are expressly included in the present invention. The compounds of this invention may also be represented in multiple tautomeric forms, in such instances, the invention expressly includes all tautomeric forms of the compounds described herein (e.g., alkylation of a ring system may result in alkylation at multiple sites, the invention expressly includes all such reaction products). All such isomeric forms of such compounds are expressly included in the present invention. All crystal forms of the compounds described herein are expressly included in the present invention.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, as hereinabove recited, or an appropriate fraction thereof, of the administered ingredient.

The dosage regimen for treating a disorder or a disease with the Wnt signaling enhancing compounds of this invention and/or compositions of this invention is based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely using standard methods.

The amounts and dosage regimens administered to a subject will depend on a number of factors, such as the mode of administration, the nature of the condition being treated, the body weight of the subject being treated and the judgment of the prescribing physician.

The amount of compound included within therapeutically active formulations according to the present invention is an effective amount for treating the disease or condition.

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In general, a therapeutically effective amount of the present preferred compound in dosage form usually ranges from slightly less than about 0.025 mg/kg/day to about 2.5 g/kg/day, preferably about 0.1 mg/kg/day to about 100 mg/kg/day of the patient or considerably more, depending upon the compound used, the condition or infection treated and the route of administration, although exceptions to this dosage range may be contemplated by the present invention. In its most preferred form, compounds according to the present invention are administered in amounts ranging from about 1 mg/kg/day to about 100 mg/kg/day. The dosage of the compound will depend on the condition being treated, the particular compound, and other clinical factors such as weight and condition of the patient and the route of administration of the compound. It is to be understood that the present invention has application for both human and veterinary use.

For oral administration to humans, a dosage of between approximately 0.1 to 100 mg/kg/day, preferably between approximately 1 and 100 mg/kg/day, is generally sufficient.

Where drug delivery is systemic rather than topical, this dosage range generally produces effective blood level concentrations of active compound ranging from less than about 0.04 to about 400 micrograms/cc or more of blood in the patient.

The compound is conveniently administered in any suitable unit dosage form, including but not limited to one containing 1 to 3000 mg, preferably 5 to 500 mg of active ingredient per unit dosage form. An oral dosage of 10-250 mg is usually convenient.

The concentration of active compound in the drug composition will depend on absorption, distribution, inactivation, and excretion rates of the drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

In certain embodiments, the compound is administered once daily; in other embodiments, the compound is administered twice daily; in yet other embodiments, the

compound is administered once every two days, once every three days, once every four days, once every five days, once every six days, once every seven days, once every two weeks, once every three weeks, once every four weeks, once every two months, once every six months, or once per year. The dosing interval can be adjusted according to the needs of individual patients. For longer intervals of administration, extended release or depot formulations can be used.

The compounds of the invention can be used to treat diseases and disease conditions that are acute, and may also be used for treatment of chronic conditions. In certain embodiments, the compounds of the invention are administered for time periods exceeding two weeks, three weeks, one month, two months, three months, four months, five months, six months, one year, two years, three years, four years, or five years, ten years, or fifteen years; or for example, any time period range in days, months or years in which the low end of the range is any time period between 14 days and 15 years and the upper end of the range is between 15 days and 20 years (e.g., 4 weeks and 15 years, 6 months and 20 years). In some cases, it may be advantageous for the compounds of the invention to be administered for the remainder of the patient's life. In preferred embodiments, the patient is monitored to check the progression of the disease or disorder, and the dose is adjusted accordingly. In preferred embodiments, treatment according to the invention is effective for at least two weeks, three weeks, one month, two months, three months, four months, five months, six months, one year, two years, three years, four years, or five years, ten years, fifteen years, twenty years, or for the remainder of the subject's life.

Still other objects, features, and attendant advantages of the present invention will become apparent to those skilled in the art from a reading of the preceding detailed description of embodiments constructed in accordance therewith, taken in conjunction with the accompanying drawings.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptide of the present invention may be employed on conjunction with other therapeutic compounds.

The invention provides for pharmaceutical compositions containing at least one Wnt signaling activator described herein. In embodiments, the pharmaceutical compositions contain a pharmaceutically acceptable carrier, excipient, or diluent, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to a subject receiving the composition, and which may be administered without undue toxicity. As used herein, the term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopia, European Pharmacopia or other generally recognized pharmacopia for use in mammals, and more particularly in humans. These compositions can be useful for treating and/or preventing viral infection and/or autoimmune disease.

A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in *Remington's Pharmaceutical Sciences* (17th ed., Mack Publishing Company) and *Remington: The Science and Practice of Pharmacy* (21st ed., Lippincott Williams & Wilkins), which are hereby incorporated by reference. The formulation of the pharmaceutical composition should suit the mode of administration. In embodiments, the pharmaceutical composition is suitable for administration to humans, and can be sterile, non-particulate and/or non-pyrogenic.

Pharmaceutically acceptable carriers, excipients, or diluents include, but are not limited, to saline, buffered saline, dextrose, water, glycerol, ethanol, sterile isotonic aqueous buffer, and combinations thereof.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives, and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include, but are not limited to: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

In embodiments, the pharmaceutical composition is provided in a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder.

In embodiments, the pharmaceutical composition is supplied in liquid form, for example, in a sealed container indicating the quantity and concentration of the active ingredient in the pharmaceutical composition. In related embodiments, the liquid form of the pharmaceutical composition is supplied in a hermetically sealed container.

Methods for formulating the pharmaceutical compositions of the present invention are conventional and well known in the art (see Remington and Remington's). One of skill in the art can readily formulate a pharmaceutical composition having the desired characteristics (e.g., route of administration, biosafety, and release profile).

Methods for preparing the pharmaceutical compositions include the step of bringing into association the active ingredient with a pharmaceutically acceptable carrier and, optionally, one or more accessory ingredients. The pharmaceutical compositions can be prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product. Additional methodology for preparing the pharmaceutical compositions, including the preparation of multilayer dosage forms, are described in *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems* (9th ed., Lippincott Williams & Wilkins), which is hereby incorporated by reference.

Pharmaceutical compositions suitable for oral administration can be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound(s) described herein, a derivative thereof, or a pharmaceutically acceptable salt or prodrug thereof as the active ingredient(s). The active ingredient can also be administered as a bolus, electuary, or paste.

In solid dosage forms for oral administration (e.g., capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more

pharmaceutically acceptable carriers, excipients, or diluents, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets, and pills, the pharmaceutical compositions can also comprise buffering agents. Solid compositions of a similar type can also be prepared using fillers in soft and hard-filled gelatin capsules, and excipients such as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet can be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets can be prepared using binders (for example, gelatin or hydroxypropylmethyl cellulose), lubricants, inert diluents, preservatives, disintegrants (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-actives, and/ or dispersing agents. Molded tablets can be made by molding in a suitable machine a mixture of the powdered active ingredient moistened with an inert liquid diluent.

The tablets and other solid dosage forms, such as dragees, capsules, pills, and granules, can optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the art.

In some embodiments, in order to prolong the effect of an active ingredient, it is desirable to slow the absorption of the compound from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the active ingredient then depends upon its rate of dissolution which, in turn, can depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered active ingredient is accomplished by dissolving or suspending the compound in an oil

vehicle. In addition, prolonged absorption of the injectable pharmaceutical form can be brought about by the inclusion of agents that delay absorption such as aluminum monostearate and gelatin.

Controlled release parenteral compositions can be in form of aqueous suspensions, microspheres, microcapsules, magnetic microspheres, oil solutions, oil suspensions, emulsions, or the active ingredient can be incorporated in biocompatible carrier(s), liposomes, nanoparticles, implants or infusion devices.

Materials for use in the preparation of microspheres and/or microcapsules include biodegradable/bioerodible polymers such as polyglactin, poly-(isobutyl cyanoacrylate), poly(2-hydroxyethyl-L-glutamine) and poly(lactic acid).

Biocompatible carriers which can be used when formulating a controlled release parenteral formulation include carbohydrates such as dextrans, proteins such as albumin, lipoproteins or antibodies.

Materials for use in implants can be non-biodegradable, e.g., polydimethylsiloxane, or biodegradable such as, e.g., poly(caprolactone), poly(lactic acid), poly(glycolic acid) or poly(ortho esters).

In embodiments, the active ingredient(s) are administered by aerosol. This is accomplished by preparing an aqueous aerosol, liposomal preparation, or solid particles containing the compound. A nonaqueous (*e.g.,* fluorocarbon propellant) suspension can be used. The pharmaceutical composition can also be administered using a sonic nebulizer, which would minimize exposing the agent to shear, which can result in degradation of the compound.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the active ingredient(s) together with conventional pharmaceutically-acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

Dosage forms for topical or transdermal administration of an active ingredient(s) includes powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active ingredient(s) can be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants as appropriate.

Transdermal patches suitable for use in the present invention are disclosed in *Transdermal Drug Delivery: Developmental Issues and Research Initiatives* (Marcel Dekker Inc., 1989) and U.S. Pat. Nos. 4,743,249, 4,906,169, 5,198,223, 4,816,540, 5,422,119, 5,023,084, which are hereby incorporated by reference. The transdermal patch can also be any transdermal patch well known in the art, including transscrotal patches. Pharmaceutical compositions in such transdermal patches can contain one or more absorption enhancers or skin permeation enhancers well known in the art (*see*, *e.g.*, U.S. Pat. Nos. 4,379,454 and 4,973,468, which are hereby incorporated by reference). Transdermal therapeutic systems for use in the present invention can be based on iontophoresis, diffusion, or a combination of these two effects.

Transdermal patches have the added advantage of providing controlled delivery of active ingredient(s) to the body. Such dosage forms can be made by dissolving or dispersing the active ingredient(s) in a proper medium. Absorption enhancers can also be used to increase the flux of the active ingredient across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the active ingredient(s) in a polymer matrix or gel.

Such pharmaceutical compositions can be in the form of creams, ointments, lotions, liniments, gels, hydrogels, solutions, suspensions, sticks, sprays, pastes, plasters and other kinds of transdermal drug delivery systems. The compositions can also include pharmaceutically acceptable carriers or excipients such as emulsifying agents, antioxidants, buffering agents, preservatives, humectants, penetration enhancers, chelating agents, gel-forming agents, ointment bases, perfumes, and skin protective agents.

Examples of emulsifying agents include, but are not limited to, naturally occurring gums, e.g. gum acacia or gum tragacanth, naturally occurring phosphatides, e.g. soybean lecithin and sorbitan monooleate derivatives.

Examples of antioxidants include, but are not limited to, butylated hydroxy anisole (BHA), ascorbic acid and derivatives thereof, tocopherol and derivatives thereof, and cysteine.

Examples of preservatives include, but are not limited to, parabens, such as methyl or propyl p-hydroxybenzoate and benzalkonium chloride.

Examples of humectants include, but are not limited to, glycerin, propylene glycol, sorbitol and urea.

Examples of penetration enhancers include, but are not limited to, propylene glycol, DMSO, triethanolamine, N,N-dimethylacetamide, N,N-dimethylformamide, 2-pyrrolidone and derivatives thereof, tetrahydrofurfuryl alcohol, propylene glycol, diethylene glycol monoethyl or monomethyl ether with propylene glycol monolaurate or methyl laurate, eucalyptol, lecithin, Transcutol®, and Azone®.

Examples of chelating agents include, but are not limited to, sodium EDTA, citric acid and phosphoric acid.

Examples of gel forming agents include, but are not limited to, Carbopol, cellulose derivatives, bentonite, alginates, gelatin and polyvinylpyrrolidone.

In addition to the active ingredient(s), the ointments, pastes, creams, and gels of the present invention can contain excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons, and volatile unsubstituted hydrocarbons, such as butane and propane.

Injectable depot forms are made by forming microencapsule matrices of compound(s) of the invention in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of compound to polymer, and the nature of the particular polymer employed, the rate of compound release can be controlled. Examples of other biodegradable polymers include

poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

Subcutaneous implants are well known in the art and are suitable for use in the present invention. Subcutaneous implantation methods are preferably non-irritating and mechanically resilient. The implants can be of matrix type, of reservoir type, or hybrids thereof. In matrix type devices, the carrier material can be porous or non-porous, solid or semi-solid, and permeable or impermeable to the active compound or compounds. The carrier material can be biodegradable or may slowly erode after administration. In some instances, the matrix is non-degradable but instead relies on the diffusion of the active compound through the matrix for the carrier material to degrade. Alternative subcutaneous implant methods utilize reservoir devices where the active compound or compounds are surrounded by a rate controlling membrane, e.g., a membrane independent of component concentration (possessing zero-order kinetics). Devices consisting of a matrix surrounded by a rate controlling membrane also suitable for use.

Both reservoir and matrix type devices can contain materials such as polydimethylsiloxane, such as Silastic™, or other silicone rubbers. Matrix materials can be insoluble polypropylene, polyethylene, polyvinyl chloride, ethylvinyl acetate, polystyrene and polymethacrylate, as well as glycerol esters of the glycerol palmitostearate, glycerol stearate, and glycerol behenate type. Materials can be hydrophobic or hydrophilic polymers and optionally contain solubilising agents.

Subcutaneous implant devices can be slow-release capsules made with any suitable polymer, e.g., as described in U.S. Pat. Nos. 5,035,891 and 4,210,644, which are hereby incorporated by reference.

In general, at least four different approaches are applicable in order to provide rate control over the release and transdermal permeation of a drug compound. These approaches are: membrane-moderated systems, adhesive diffusion-controlled systems, matrix dispersion-type systems and microreservoir systems. It is appreciated that a controlled release percutaneous and/or topical composition can be obtained by using a suitable mixture of these approaches.

In a membrane-moderated system, the active ingredient is present in a reservoir which is totally encapsulated in a shallow compartment molded from a drug-impermeable laminate,

such as a metallic plastic laminate, and a rate-controlling polymeric membrane such as a microporous or a non-porous polymeric membrane, e.g., ethylene-vinyl acetate copolymer. The active ingredient is released through the rate controlling polymeric membrane. In the drug reservoir, the active ingredient can either be dispersed in a solid polymer matrix or suspended in an unleachable, viscous liquid medium such as silicone fluid. On the external surface of the polymeric membrane, a thin layer of an adhesive polymer is applied to achieve an intimate contact of the transdermal system with the skin surface. The adhesive polymer is preferably a polymer which is hypoallergenic and compatible with the active drug substance.

In an adhesive diffusion-controlled system, a reservoir of the active ingredient is formed by directly dispersing the active ingredient in an adhesive polymer and then by, e.g., solvent casting, spreading the adhesive containing the active ingredient onto a flat sheet of substantially drug-impermeable metallic plastic backing to form a thin drug reservoir layer.

A matrix dispersion-type system is characterized in that a reservoir of the active ingredient is formed by substantially homogeneously dispersing the active ingredient in a hydrophilic or lipophilic polymer matrix. The drug-containing polymer is then molded into disc with a substantially well-defined surface area and controlled thickness. The adhesive polymer is spread along the circumference to form a strip of adhesive around the disc.

A microreservoir system can be considered as a combination of the reservoir and matrix dispersion type systems. In this case, the reservoir of the active substance is formed by first suspending the drug solids in an aqueous solution of water-soluble polymer and then dispersing the drug suspension in a lipophilic polymer to form a multiplicity of unleachable, microscopic spheres of drug reservoirs.

Any of the above-described controlled release, extended release, and sustained release compositions can be formulated to release the active ingredient in about 30 minutes to about 1 week, in about 30 minutes to about 72 hours, in about 30 minutes to 24 hours, in about 30 minutes to 12 hours, in about 30 minutes to 6 hours, in about 30 minutes to 4 hours, and in about 3 hours to 10 hours. In embodiments, an effective concentration of the active ingredient(s) is sustained in a subject for 4 hours, 6 hours, 8 hours, 10 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, or more after administration of the pharmaceutical compositions to the subject.

Dosages

When the agents described herein are administered as pharmaceuticals to humans or animals, they can be given per se or as a pharmaceutical composition containing active ingredient in combination with a pharmaceutically acceptable carrier, excipient, or diluent.

Actual dosage levels and time course of administration of the active ingredients in the pharmaceutical compositions of the invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. Generally, agents or pharmaceutical compositions of the invention are administered in an amount sufficient to reduce or eliminate symptoms associated with viral infection and/or autoimmune disease.

Exemplary dose ranges include 0.01 mg to 250 mg per day, 0.01 mg to 100 mg per day, 1 mg to 100 mg per day, 10 mg to 100 mg per day, 1 mg to 10 mg per day, and 0.01 mg to 10 mg per day. A preferred dose of an agent is the maximum that a patient can tolerate and not develop serious or unacceptable side effects. In embodiments, the agent is administered at a concentration of about 10 micrograms to about 100 mg per kilogram of body weight per day, about 0.1 to about 10 mg/kg per day, or about 1.0 mg to about 10 mg/kg of body weight per day.

In embodiments, the pharmaceutical composition comprises an agent in an amount ranging between 1 and 10 mg, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg.

In embodiments, the therapeutically effective dosage produces a serum concentration of an agent of from about 0.1 ng/ml to about 50-100 µg/ml. The pharmaceutical compositions typically should provide a dosage of from about 0.001 mg to about 2000 mg of compound per kilogram of body weight per day. For example, dosages for systemic administration to a human patient can range from 1-10 µg/kg, 20-80 µg/kg, 5-50 µg/kg, 75-150 µg/kg, 100-500 µg/kg, 250-750 µg/kg, 500-1000 µg/kg, 1-10 mg/kg, 5-50 mg/kg, 25-75 mg/kg, 50-100 mg/kg, 100-250 mg/kg, 50-100 mg/kg, 250-500 mg/kg, 500-750 mg/kg, 750-1000 mg/kg, 1000-1500 mg/kg, 1500-2000 mg/kg, 5 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 500 mg/kg, 1000 mg/kg, 1500 mg/kg, or 2000 mg/kg. Pharmaceutical dosage unit forms are prepared to provide from about 1 mg to about 5000 mg, for example from about 100 to about 2500 mg of the compound or a combination of essential ingredients per dosage unit form.

In embodiments, about 50 nM to about 1μM of an agent is administered to a subject. In related embodiments, about 50-100 nM, 50-250 nM, 100-500 nM, 250-500 nM, 250-750 nM, 500-750 nM, 500 nM to 1 μM, or 750 nM to 1μM of an agent is administered to a subject.

Determination of an effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. Generally, an efficacious or effective amount of an agent is determined by first administering a low dose of the agent(s) and then incrementally increasing the administered dose or dosages until a desired effect (e.g., reduce or eliminate symptoms associated with viral infection or autoimmune disease) is observed in the treated subject, with minimal or acceptable toxic side effects. Applicable methods for determining an appropriate dose and dosing schedule for administration of a pharmaceutical composition of the present invention are described, for example, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, Goodman *et al*., eds., 11th Edition, McGraw-Hill 2005, and *Remington: The Science and Practice of Pharmacy*, 20th and 21st Editions, Gennaro and University of the Sciences in Philadelphia, Eds., Lippencott Williams & Wilkins (2003 and 2005), each of which is hereby incorporated by reference.

Combination Therapies

The agents and pharmaceutical compositions described herein can also be administered in combination with another therapeutic molecule. The therapeutic molecule can be any compound used to retinal disease, or symptoms thereof. Examples of such compounds include, but are not limited to, anti-viral agents, immunosuppressants, anti-inflammatories, and the like.

The Wnt signaling enhancing compounds can be administered before, during, or after administration of the additional therapeutic agent. In embodiments, the Wnt signaling enhancing compounds is administered before the first administration of the additional therapeutic agent. In embodiments, the Wnt signaling enhancing compounds is administered after the first administration of the additional therapeutic agent (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 days or more). In embodiments, the Wnt signaling enhancing compounds is administered simultaneously with the first administration of the additional therapeutic agent.

The amount of therapeutic agent administered to a subject can readily be determined by the attending physician or veterinarian. Generally, an efficacious or effective amount of an Wnt signaling enhancing compound and an additional therapeutic is determined by first administering a low dose of one or both active agents and then incrementally increasing the administered dose or dosages until a desired effect is observed (e.g., reduced symptoms associated with viral infection or autoimmune disease), with minimal or no toxic side effects. Applicable methods for determining an appropriate dose and dosing schedule for administration of a combination of the present invention are described, for example, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 11th Edition., supra, and in *Remington: The Science and Practice of Pharmacy*, 20th and 21st Editions, supra.

Kits

The invention provides for kits containing at least one Wnt signaling enhancing compound as described herein. The kits are suitable for use in preventing or treating capillary dropout associated with retinal disease. In embodiments, the Wnt signaling enhancing compounds is provided as a pharmaceutical composition. In embodiments, the kit provides instructions for use. The instructions for use can pertain to any of the methods described herein.

Kits according to this aspect of the invention may comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampules, bottles and the like. In embodiments, the kit provides a notice in the form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale of the kit and the components therein for human administration.

EXAMPLES

It should be appreciated that the invention should not be construed to be limited to the examples that are now described; rather, the invention should be construed to include any and all applications provided herein and all equivalent variations within the skill of the ordinary artisan.

Example 1: Identification of optimal time to initiate treatment of capillary dropout.

Capillary drop out is a very serious problem for many retinal vascular diseases. It has been previously suggested that norrin can be used to treat capillary dropout. *See* Trese, M. T. *et*

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al., *Retinal Physician*, April 2011. For example, in a murine capillary dropout model (*see* Figures 1 and 2; *see also* Figure 3), capillary dropout is reduced or eliminated by norrin treatment (*see* Figure 4).

It has now been discovered that there is a peripheral vascular change on wide field angiography that proceeds capillary loss. This antecedent lesion is characterized by the presence of vessels having fuzzy margins during fluorescein angiograms. *See* Figures 5, 6, and 7. Specifically, Figure 5 is a wide field fluorescein angiogram (Optos system) of a person with Familial Exudative Vitreoretinopathy (FEVR). This patient previously received laser therapy to treat avascular retina (as shown by the retinal damage present on the far right of the image). Health optical nerve is present throughout the left half of the image. Between the healthy optical nerve and the retinal damage from the laser therapy is a region of "fuzzy" vessels (i.e., where vessel diameter is increased with indistinct margins in a fundus image as compared to the remaining vessels, which maintain a crisp architecture). These fuzzy vessels are an indication of cellular change due to damage of endothelial cells. These changes precede frank capillary loss, which is what drives the pathogenesis of many retinal disorders. Illustratively, Figure 6 depicts a higher magnification view of the area of capillary damage shown in FIG. 5. Shown are small areas of microanyeurisms (i.e., punctate white spots) and endothelial cell damage as manifested by dye leakage. Left alone this area will ultimately result in complete capillary loss.

Additionally, Figure 7 shows a low magnification wide field fluorescein angiogram (Optos) of a retina of a patient with diabetes prior to the onset of macular diabetic retinopathy (e.g., either background diabetic retinopathy or macular diabetic retinopathy), which reveals numerous small white dots that correspond to microaneurisms surrounding areas of capillary drop out prior to any change in the macula area. These microaneurism-associated areas are believed to give rise to macular diabetic retinopathy, and according to an exemplary embodiment of the invention, these areas represent target areas for treatment with an activator of Wnt signaling (e.g., Norrin). Figures 8 shows a high magnification view of the wide field fluorescein angiogram shown in FIG. 7. In comparison, Figures 9 and 10 show regular and wide field, respectively, fluorescein angiogram depicting normal macula.

This finding is of great clinical significance because it will assist practitioners in accurately identifying and effectively treating early stage retinal disease. Early detection and early treatment of capillary loss will result in preservation and/or reformation of the altered vessels.

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Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Incorporation by Reference

All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

References:

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Planutis, K, et al, BMC Cell Biology, 2007; 8:12.

What is claimed is:

1. A method of treating or preventing capillary dropout in a subject, wherein the method comprises administering to the subject an agent that enhances Wnt signaling, thereby treating or preventing capillary dropout.

2. The method of claim 1, wherein the subject has or is at risk of developing capillary dropout.

3. The method of claim 2, wherein the subject has or is at risk of developing familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), Norries disease, branch retinal vein occlusion, or central retinal vein occlusion.

4. The method of claim 2, wherein the subject has or is at risk of developing diabetic retinopathy.

5. The method of any one of claims 1 to 3, wherein the ocular capillaries of the subject are evaluated by fluorescein angiography (FA).

6. The method of claim 5, wherein the agent is administered to the subject when an antecedent lesion is identified.

7. The method of claim 6, wherein the antecedent lesion is located in a peripheral capillary.

8. The method of claim 6, wherein the antecedent lesion is located in a posterior capillary.

9. A method of treating or preventing peripheral capillary dropout in a subject, wherein the method comprises administering to the subject an agent that enhances Wnt signaling, thereby treating or preventing capillary dropout.

10. The method of claim 9, wherein the subject has or is at risk of familial exudative vitreoretinopathy (FEVR), diabetic retinopathy, retinopathy of prematurity (ROP), Norries disease, branch retinal vein occlusion, or central retinal vein occlusion.

11. The method of claim 9, wherein the subject has or is at risk of developing diabetic retinopathy.

12. The method of any one of claims 9 to 11, wherein the ocular capillaries of the subject are evaluated by fluorescein angiography (FA).

13. The method of claim 12, wherein the agent is administered to the subject when an antecedent lesion is identified in a peripheral capillary.

14. The method of any one of claims 1 to 13, wherein the method reduces capillary loss.

15. The method of any one of claims 1 to 14, wherein the method reduces capillary occlusion.

16. The method of any one of claims 1 to 15, wherein the method enhances capillary formation.

17. The method of any one of claims 1 to 16, wherein the method enhances retinal function.

18. The method of any one of claims 1 to 17, wherein the method enhances b-wave response, substantial oscillatory potential, or visual improvement.

19. The method of any one of claims 1 to 18, wherein the method reduces the symptoms associated with capillary dropout.

20. The method of any one of claims 1 to 19, wherein the agent enhances the activity of a frizzled-4 transmembrane receptor (Fzd4).

21. The method of any one of claims 1 to 20, wherein the agent is an agonist of Fzd4, Low-density lipoprotein receptor-related protein 5 (LRP5), Low-density lipoprotein receptor-related protein 6 (LRP6), or tetraspanin-12 (Tspan12).

22. The method of any one of claims 1 to 21, wherein the agent is a small molecule, a nucleic acid, a peptide, or a peptide mimetic.

23. The method of claim 22, wherein the agent is an antibody or antibody fragment.

24. The method of claim 22, wherein the small molecule is 2-amino-4-[3,4-(methylenedioxy)benzyl-amino]-6-(3-methoxyphenyl)pyrimidine.

25. The method of claim 23, wherein the antibody is a polyclonal antibody.

26. The method of claim 23, wherein the antibody is a monoclonal antibody.

27. The method of any one of claims 23 to 26, wherein the antibody or antibody fragment is humanized.

28. The method of any one of claims 1 to 22, wherein the agent is a ligand of Fzd4.

29. The method of claim 28, wherein the ligand is Norrin, an active fragment of Norrin, or an active mutant of Norrin.

30. The method of any one of claims 1 to 22, wherein the agent is a nucleic acid encoding Norrin or a fragment thereof.

31. A method of treating or preventing capillary dropout in a subject having or at risk of developing diabetic retinopathy, wherein the method comprises administering Norrin or a fragment thereof to the subject, thereby treating or preventing capillary dropout.

32. The method of any one of claims 1 to 31, wherein the method further comprises administering at least one additional agent to treat capillary dropout.

33. The method of claim 31, wherein the at least one additional agent is an anti-inflammatory, a steroid, anti-VEGF, or a caspase.

34. The method of any one of claims 1 to 33, wherein the method further comprises treating the patient with laser therapy.

35. The method of any one of claims 1 to 34, wherein the agent is administered systemically or locally.

36. The method of claim 35, wherein the agent is administered orally, parenterally, or topically.

37. The method of claim 35 or 36, wherein the agent is administered by intravitreal or periocular injection.

38. The method of any one of claims 1 to 37, wherein the subject is a mammal.

39. The method of claim 38, wherein the subject is human.

40. A pharmaceutical composition comprising an agent that treats capillary dropout for use in any one of the methods of claims 1 to 39.

41. The pharmaceutical composition of claim 40, wherein the agent that treats capillary dropout enhances the activity of a frizzled-4 transmembrane receptor (Fzd4).

42. The pharmaceutical composition of claim 40 or 41, wherein the agent that treats capillary dropout is an agonist of Fzd4, Low-density lipoprotein receptor-related protein 5 (LRP5), or tetraspanin-12 (Tspan12).

43. The pharmaceutical composition of any one of claims 40 to 42, wherein the agent that treats capillary dropout is a small molecule, a nucleic acid, a peptide, or a peptide mimetic.

44. The pharmaceutical composition of claim 43, wherein the agent that treats capillary dropout is an antibody or antibody fragment.

45. The pharmaceutical composition of claim 44, wherein the agent that treats capillary dropout is a polyclonal antibody.

46. The pharmaceutical composition of claim 44, wherein the agent that treats capillary dropout is a monoclonal antibody.

47. The pharmaceutical composition of any one of claims 44 to 46, wherein the antibody or antibody fragment is humanized.

48. The pharmaceutical composition of any one of claims 40 to 43, wherein the agent that treats capillary dropout is a ligand of Fzd4.

49. The pharmaceutical composition of claim 48, wherein the ligand is Norrin or a fragment thereof.

50. The pharmaceutical composition of any one of claims 40 to 43, wherein the agent that treats capillary dropout is a nucleic acid encoding Norrin or a fragment thereof.

51. The pharmaceutical composition of claim any one of claims 40 to 50, wherein the pharmaceutical composition further comprises at least one additional agent to treat capillary dropout.

52. The pharmaceutical composition of claim any one of claims 40 to 51, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier, diluent, or excipient.

53. A kit comprising an agent that treats capillary dropout for use in any one of the methods of claims 1 to 39.

54. The kit of claim 53, wherein the agent that treats capillary dropout enhances the activity of a frizzled-4 transmembrane receptor (Fzd4).

55. The kit of claim 53 or 54, wherein the agent that treats capillary dropout is an agonist of Fzd4, Low-density lipoprotein receptor-related protein 5 (LRP5), or tetraspanin-12 (Tspan12).

56. The kit of any one of claims 53 to 55, wherein the agent that treats capillary dropout is a small molecule, a nucleic acid, a peptide, or a peptide mimetic.

57. The kit of claim 56, wherein the agent that treats capillary dropout is an antibody or antibody fragment.

58. The kit of claim 57, wherein the agent that treats capillary dropout is a polyclonal antibody.

59. The kit of claim 57, wherein the agent that treats capillary dropout is a monoclonal antibody.

60. The kit of any one of claims 57 to 59, wherein the antibody or antibody fragment is humanized.

61. The kit of any one of claims 53 to 56, wherein the agent that treats capillary dropout is a ligand of Fzd4.

62. The kit of claim 61, wherein the ligand is Norrin or a fragment thereof.

63. The kit of any one of claims 53 to 56, wherein the agent that treats capillary dropout is a nucleic acid encoding Norrin or a fragment thereof.

64. The kit of any one of claims 53 to 63, wherein the kit further comprises at least one additional agent to treat capillary dropout.

65. The kit of any one of claims 53 to 64, wherein the kit further comprises instructions for using the agent that treats capillary dropout in any one of the methods of claims 1 to 39.

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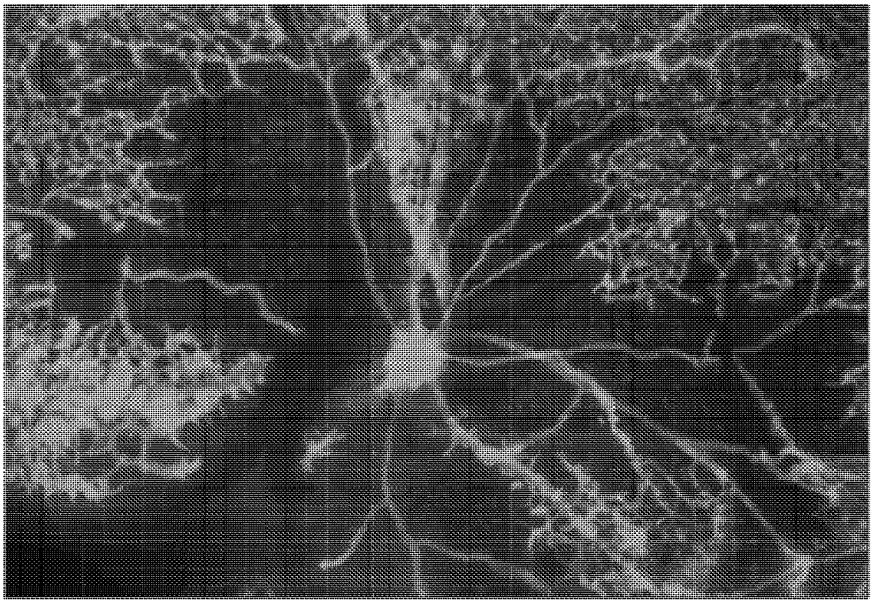


FIG. 1

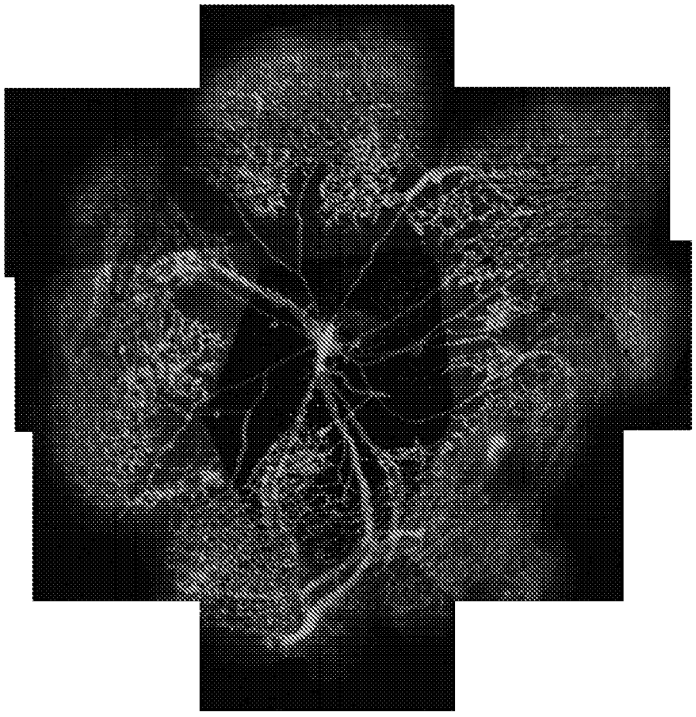


FIG. 2

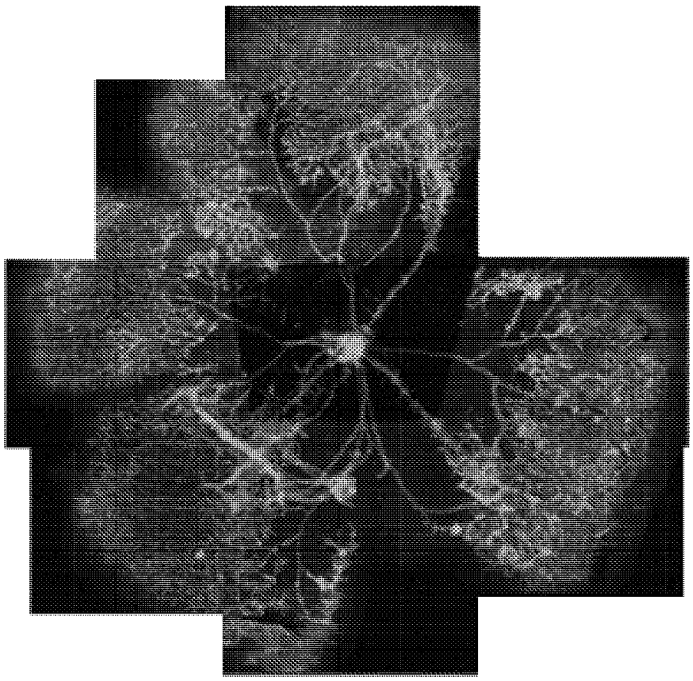


FIG. 3

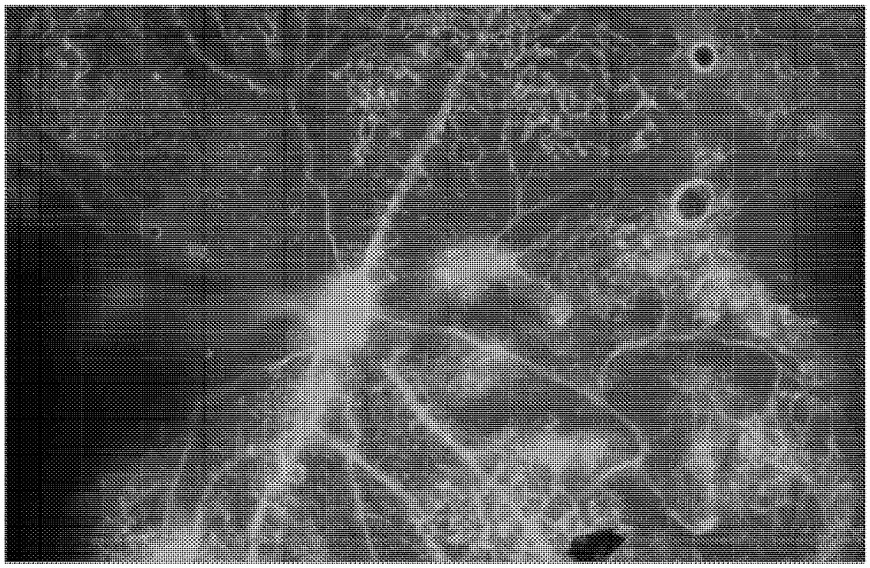


FIG. 4

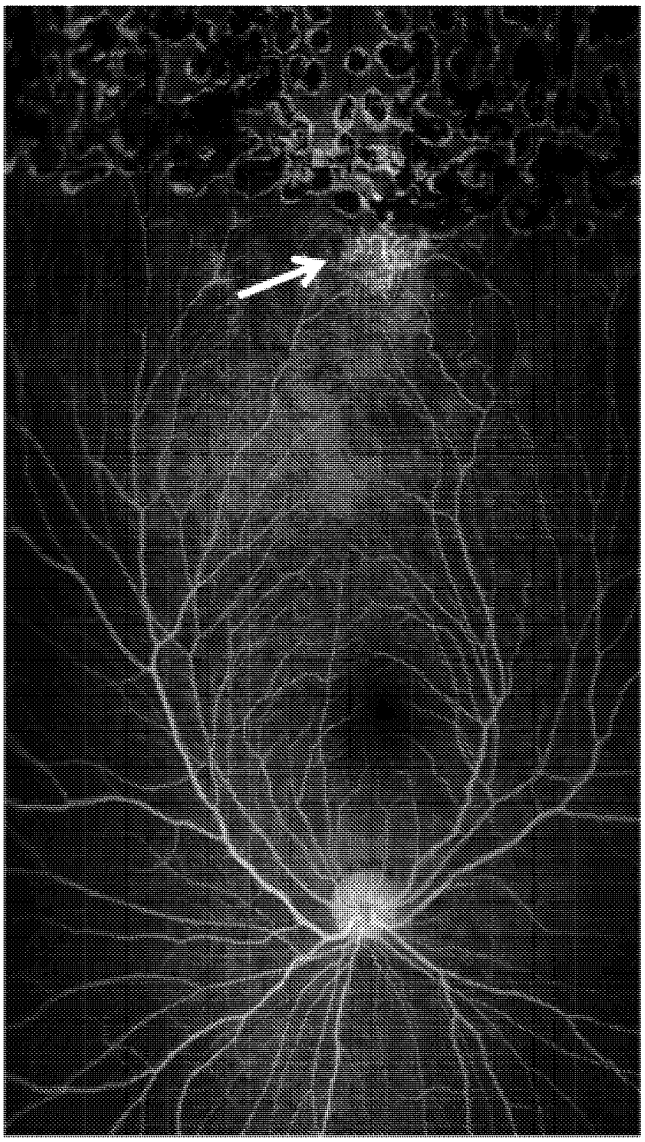


FIG. 5

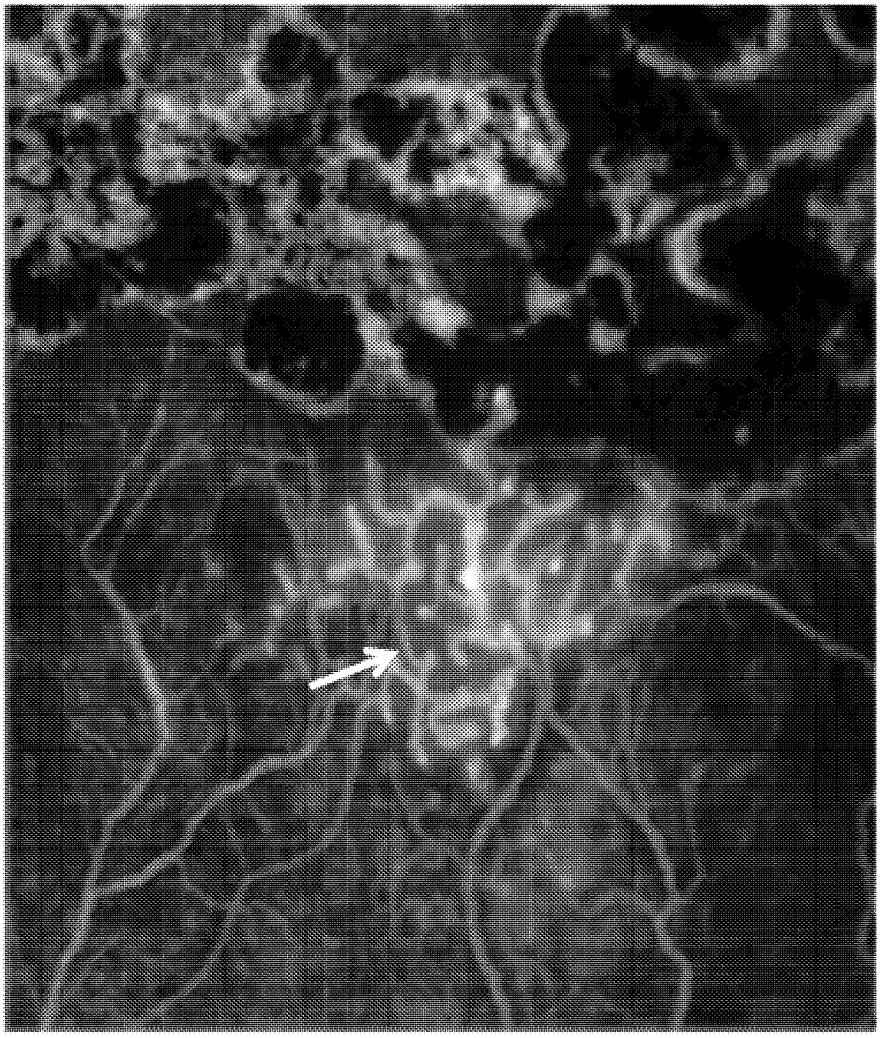


FIG. 6

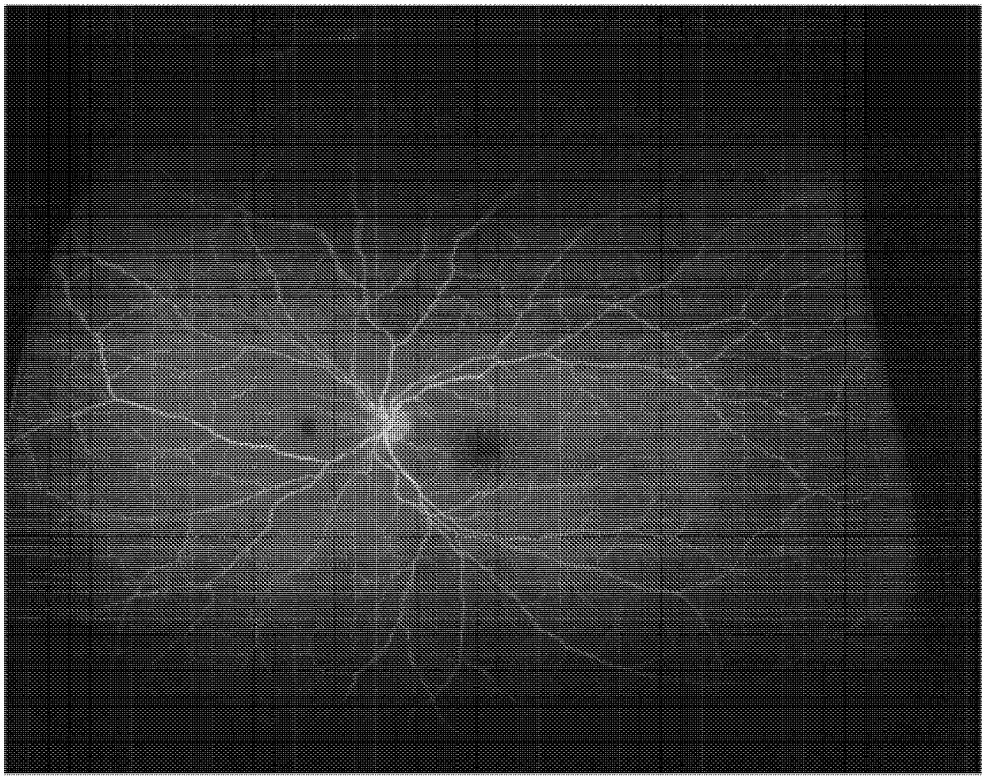


FIG. 7

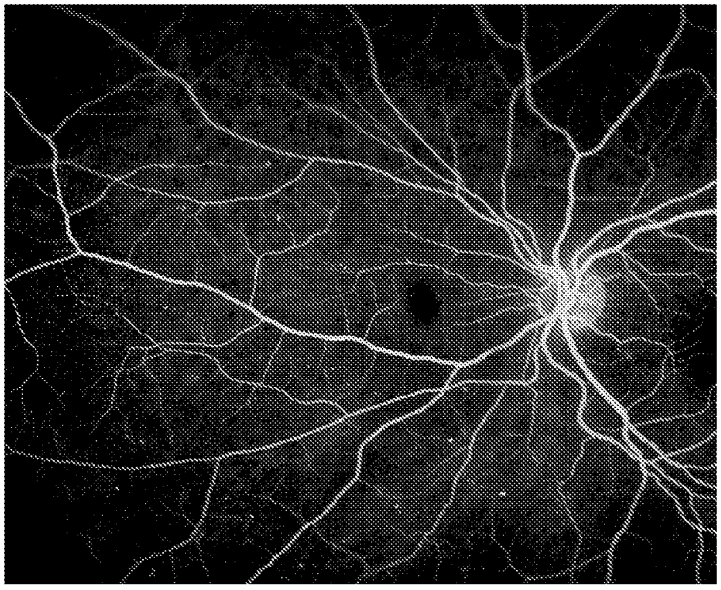
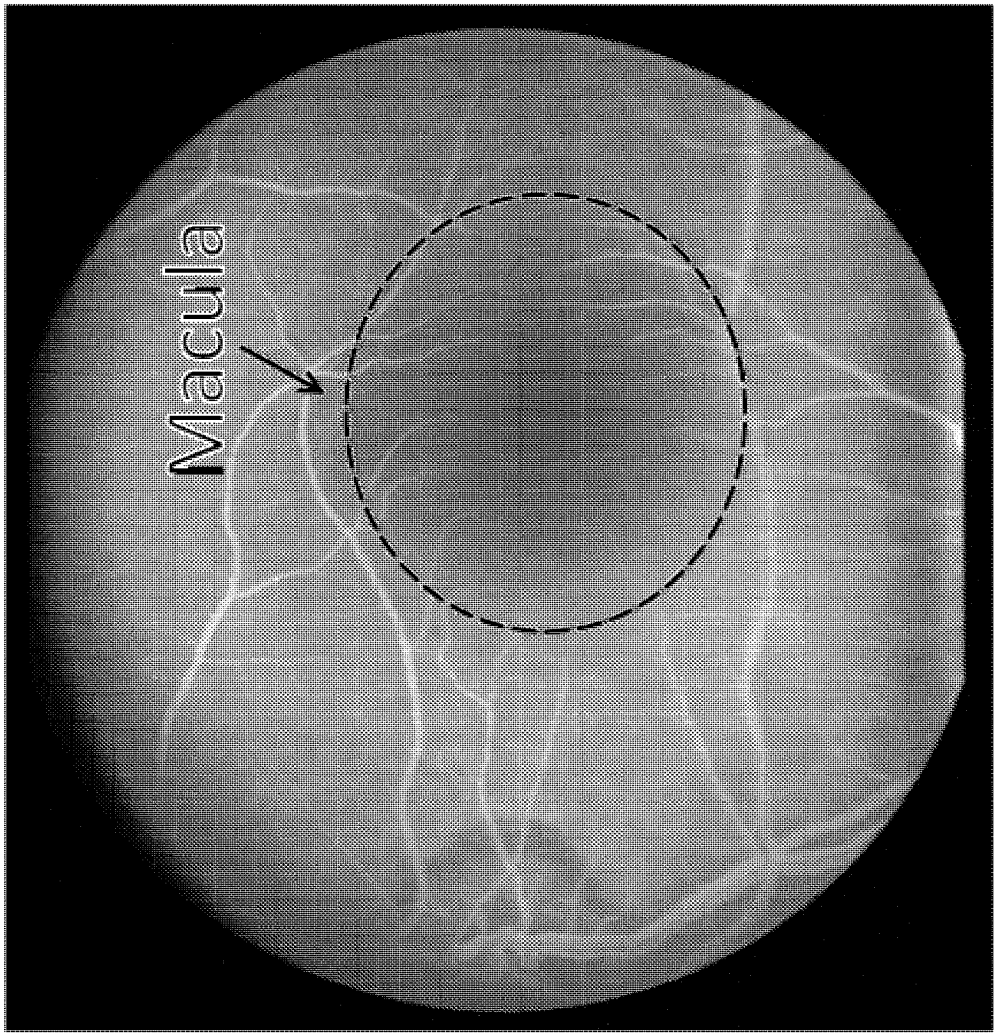


FIG. 8

FIG. 9



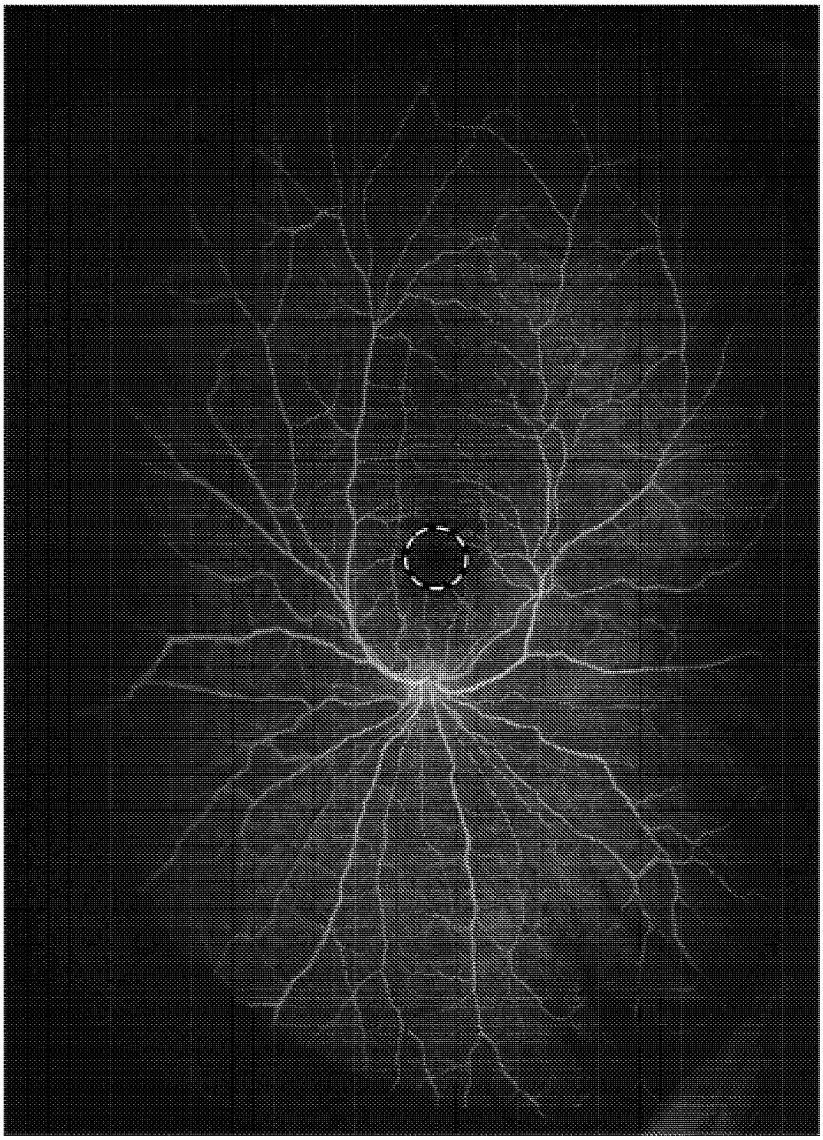


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032411

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/17(2006.01)i, A61K 38/16(2006.01)i, A61K 48/00(2006.01)i, A61P 27/02(2006.01)i, A61P 27/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 38/17; A61B 10/00; A61K 48/00; A61K 38/16; A61P 27/02; A61P 27/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: capillary dropout, Wnt, diabetic retinopathy, norrin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010-0239499 A1 (DRENSER) 23 September 2010 See abstract; paragraphs [0001]-[0007]; and claims 1-23.	1-4,9-11,31
A	SEITZ et al., `Norrin mediates neuroprotective effects on retinal ganglion cells via activation of the Wnt/β-catenin signaling pathway and the induction of neuroprotective growth factors in Muller cells` The Journal of Neuroscience, Vol.30, No.17, pp.5998-6010 (2010) See the whole document.	1-4,9-11,31
A	OHLMANN et al., `Norrin promotes vascular regrowth after oxygen-induced retinal vessel loss and suppresses retinopathy in mice` The Journal of Neuroscience, Vol.30, No.1, pp.183-193 (2010) See the whole document.	1-4,9-11,31
A	CHEN et al., `Wnt signaling mediates pathological vascular growth in proliferative retinopathy clinical perspective` Circulation, Vol.124, No.17, pp.1871-1881 (2011) See the whole document.	1-4,9-11,31

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 2013 (22.10.2013)	**22 October 2013 (22.10.2013)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. +82-42-472-7140	Authorized officer KIM, Seung Beom Telephone No. +82-42-481-3371

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032411

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MASCKAUCHAN et al., `Wnt/Frizzled signaling in the vasculature: new angiogenic factors in sight` Physiology, Vol.21, No.3, pp.181-188 (2006) See the whole document.	1-4,9-11,31

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032411

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: See extra sheet
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See extra sheet

3. ☒ Claims Nos.: See extra sheet
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

(Continuation of: Box No. II)

2. Claims 5-8, 12-13, 23-26, 29, 33, 36, 39, 41, 44-46, 49, 54, 57-59, 62

Claims 5 and 12 are worded in reference to the ocular capillaries of any one of claims 1-3 and any one of claims 9-11, respectively. However, said ocular capillaries have not been worded in any one of claims 1-3 and 9-11. Therefore, claims 5 and 12 do not meet the requirement of PCT Article 6. Claims 6-8 and 13 do not comply with PCT Article 6 because they are referring to unsearchable claims 5 and 12, respectively.

Claims 23-26, 29, 36, 39, 41, 44-46, 49, 54, 57-59 and 62 are unclear since they are referring to multiple dependent claims which do not comply with PCT Rule 6.4(a).

Claim 33 is worded in reference to at least one additional agent of claim 31. However, said at least one additional agent has not been worded in claim 31. Therefore, claim 33 does not meet the requirement of PCT Article 6.

3. Claims 14-22, 27-28, 30, 32, 34-35, 37-38, 40, 42-43, 47-48, 50-53, 55-56, 60-61, 63-65

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010-0239499 A1	23/09/2010	None	

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(54) Title: WNT-SURROGATE AGENTS AND METHODS FOR LACRIMAL GLAND REGENERATION

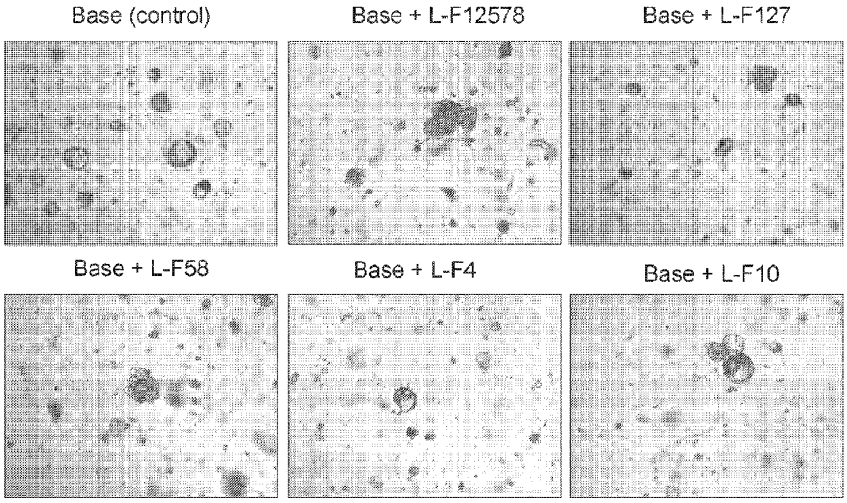


FIG. 1

(57) Abstract: Methods and compositions for treatment of dry-eye disorders are provided. In particular, the present disclosure provides methods and compositions for modulating WNT (Wingless and Int-1) signaling in a subject to modulate regeneration of tear-producing acinar cells to treat aqueous-deficient type dry-eye diseases. Further disclosed are WNT signaling modulators used in the methods.

[Continued on next page]

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TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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WNT-SURROGATE AGENTS AND METHODS FOR LACRIMAL GLAND REGENERATION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/291,243 filed on December 17, 2021, each of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

[0002] The Sequence Listing XML associated with this application is provided in XML file format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing XML is SRZN_024_01WO_ST26.xml. The XML file is 61,424 bytes, was created on December 15, 2022, and is being submitted electronically via USPTO Patent Center.

FIELD OF INVENTION

[0003] The present disclosure relates to WNT signal modulators and treatment methods for various ophthalmology associated disorders.

BACKGROUND OF THE INVENTION

[0004] The lacrimal gland is an exocrine gland positioned under the outer lateral portion of the upper eyelid in most vertebrates. The lacrimal gland produces a tear film, which is secreted by lacrimal ducts, and is associated with ocular health. (*See* Yao & Zhang at 939.) The film keeps the cornea surface and inner eyelids moistened and protects the corneal and conjunctival epithelia from physical damage, as well as from immune reactions. (*See id.*)

[0005] Lacrimal gland disorders and damage can cause significant ocular disease pathologies, especially dry-eye disease. (Dartt, OCULAR DISEASE at 105) Dry-eye disease is characterized by increased osmolarity of the tear film, and inflammation of the ocular surface. (*See id.*) Inadequate production of tears can lead to irritation, pain, and potentially damage to the ocular surface.

[0006] There are two major classes of dry-eye diseases: aqueous-deficient and evaporative. In turn, aqueous deficiency is subclassed into Sjögren's syndrome (autoimmune) dry-eye and non-Sjögren's syndrome (non-autoimmune) dry-eye.

[0007] In cases of aqueous-deficient dry-eye, the lacrimal gland is frequently inflamed, leading to atrophy and cell death of the tear-fluid-producing acinar cells. Following loss of acinar cells, tear production problems worsen leading to a reinforcing vicious inflammation-atrophy cycle. Accordingly, the lacrimal gland is a target in aqueous-deficient dry-eye diseases.

[0008] Presently, both aqueous-deficient and evaporative dry-eye diseases are treated by topical administration on the ocular surface of artificial tears and/or anti-inflammatory agents. (*See id.* at 106.) Only a few treatments target the lacrimal gland itself, by systemic administration of anti-inflammatory agents, so-called secretagogue agents, immunosuppressive agents, and sex hormones. (*See id.*) None of these treatment approaches deal directly with atrophy and/or damage to the lacrimal gland, including the tear-producing acinar cells. Consequently, there is a great unmet need for agents and methods for regeneration of acinar cells, restoring endogenous tear production and breaking the cycle of inflammation and atrophy.

BRIEF SUMMARY

[0009] The present description provides methods of treating dry-eye disorders by activation and/or regeneration of lacrimal gland and/or lacrimal gland acinar cells, and compositions therefor.

[0010] In an aspect, the present invention comprises a method of regenerating lacrimal gland acinar cells, progenitor cells, ductal cells, myoepithelial cells, or immune cells in a subject, comprising administering a WNT signaling modulator to the subject. In an embodiment, the WNT signaling modulator may be an engineered WNT signaling modulator. In another embodiment, the WNT signaling modulator is an engineered WNT agonist or superagonist, or an engineered WNT antagonist. In particular embodiments, the cells are epithelial stem and/or progenitor cells, e.g., lacrimal gland epithelial stem and/or progenitor cells.

[0011] In an embodiment of the method of regenerating lacrimal gland acinar cells, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. In any embodiment, the engineered WNT agonist may be selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic. The WNT mimetic may be a SWAP™ compound. The R-spondin mimetic may be a SWEETS™ compound.

[0012] In an embodiment of the method of regenerating lacrimal gland acinar cells, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8, and Lrp6, and/or Lrp5. For example, the WNT signaling modulator may target any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; or any one or more of the group consisting of: Fzd5 and Fzd8, while also targeting Lrp6 and/or Lrp5. For another example, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, and Fzd7.

[0013] In one embodiment of the method of regenerating lacrimal gland acinar cells, the method further comprises the step of administering at least one of the group consisting of: RSPO2, RSPO2 fragment, and engineered RSPO2 mimetic.

[0014] In any embodiment, the WNT signaling modulator may be in a concentration ≥1nM. In any embodiment, the WNT signaling modulator may be administered in a therapeutically effective amount. In any embodiment, the subject may be a live mammal. In any embodiment, the subject may be a human patient.

[0015] In another aspect, the present invention comprises a method of treating a lacrimal gland disorder in a subject, comprising administering a WNT signaling modulator to the subject. In an embodiment, the WNT signaling modulator may be an engineered WNT signaling modulator. In another embodiment, the WNT signaling modulator is an engineered WNT agonist or an engineered WNT antagonist. In further embodiment, the WNT signaling modulator is an engineered WNT superagonist.

[0016] In an embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. In any embodiment, the engineered WNT agonist may be selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic. The WNT mimetic may be a SWAP™ compound. The R-spondin mimetic may be a SWEETS™ compound.

[0017] In an embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8, and Lrp6, and/or Lrp5. For example, the WNT signaling modulator may target any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; or any one or more of the group consisting of: Fzd5 and Fzd8, while also targeting Lrp6 and/or Lrp5. For another example, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, and Fzd7.

[0018] In one embodiment of the method of treating a lacrimal gland disorder, the method further comprises the step of administering at least one of the group consisting of: RSPO2, RSPO2 fragment, and engineered RSPO2 mimetic.

[0019] In any embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may be in a concentration ≥1nM. In any embodiment, the WNT signaling modulator may be administered in a therapeutically effective amount. In any embodiment, the subject may be a live mammal. In any embodiment, the subject may be a human patient.

[0020] In still another aspect, the present invention comprises a composition for the treatment of a dry-eye disorder in a subject, the composition comprising a WNT signaling modulator. In any embodiment of the composition, the dry-eye disorder may be due to Sjögren's syndrome disorder, chronic graft versus host disease (cGHVD), rheumatoid arthritis (RA), Stephen's Johnson syndrome, Ocular Rosacea, chemotherapy, radiation oncology treatments, diabetes, lupus, etc.

[0021] In an embodiment of the composition, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. The at least one engineered bi-specific full-length IgG antibody may be specific for any one or more of Fzd1, Fzd,2, Fzd5, Fzd7, Fzd8, and Lrp6, and or Lrp5.

[0022] In an embodiment, the composition may further comprise an anti-inflammatory agent or lacrimal gland secretagogue.

[0023] In any embodiment of the composition, the composition may comprise therapeutically effective amounts of each of its components.

[0024] In any embodiment of the composition, the subject may be a live mammal. In any embodiment of the composition, the subject may be a human patient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Fig. 1 depicts representative optical micrograph images of organoid outgrowth from primary mouse lacrimal gland tissue after 7 days using a standard 3D organoid protocol in Advanced DMEM supplemented with additional growth factors. Conditions include Base containing R-spondin 1 (RSPO1) alone (control, upper left), or the addition of various WNT mimetic compounds, each at 5nM concentration. "F" represents various FZD binders (e.g., 18R5 - a FZD 1,2,5,7,8 binder ("F12578"); R2H1 – a FZD1,2,7 binder ("F127"); 2919 – a FZD 5,8 binder ("F58"); 5063 – a FZD4 binder ("F4"); and HB9L9.3 – a FZD10 binder

(F10)) that bind to various FZD receptors; L is an LRP binder, YW211.31.57, that binds to LRP5 and LRP6.

[0026] Fig. 2 depicts a micrograph image showing lacrimal organoid morphology (left), fluorescence indicating *Mist1* acinar cell marker gene expression (center) and composite (right), scale bar is 100 μm.

[0027] Figs. 3A-3C depict bar graphs indicating relative gene expression of either proliferative markers or markers specific for lacrimal-derived cells and acinar cells, in profiled organoids expanded for 7 days in control medium or in medium with L6-F12578 WNT mimetic compound. Measured genes include WNT target *Axin2* (Fig. 3A), lacrimal duct cell marker *Krt7* (Fig. 3B), and lacrimal acinar cell marker *Mist1* (Fig. 3C).

[0028] Fig. 4 depicts a graph quantifying organoid outgrowth by luminescence in a 7-day WNT mimetic screen in acinar cell organoid cultures. Starting material was lacrimal gland acinar cell organoid cells. Medium contained RSPO1 alone (control), or the addition of a WNT mimetic at 5nM concentration.

[0029] Fig. 5 depicts an optical micrograph showing organoid morphology at the end of WNT mimetic screen, in control (left panel) or L6-F12578 (right panel). Solid budding morphology in high WNT is shared with outgrowth from primary tissue and indicative of acinar cell identity. Scale bar is 200 μm.

[0030] Fig. 6 depicts a graph quantifying viable organoid cells by luminescence (in relative light units (RLU)) in organoid cells expanded for 7 days at several doses of L6-F127 WNT mimetic (0.05nM to 5nM) with or without RSPO1 (500 ng/mL).

[0031] Fig. 7 depicts of a schematic representation of the experimental design to measure acute WNT target gene induction at 24 hours and 48 hours post exposure.

[0032] Fig. 8 depicts a representative optical micrograph image of organoid cultures at the moment of WNT stimulus at day 7 post plating. Scale bar is 200 μm.

[0033] Fig. 9 depicts a graph quantifying WNT target *Axin2* expression levels by qPCR at 24 hours (left) and 48 hours (right) post-induction. Expression for different conditions were normalized to *actinB* expression and relative to control (no WNT mimetic).

[0034] Fig. 10 depicts an image of two non-dry-eye human lacrimal glands, which were used in explant experiments.

[0035] Figs. 11A and 11B depicts optical micrographs of human lacrimal gland cell cultures after dissociation (Fig. 11A) and after 24 hours in acinar cell medium (Fig. 11B). Scale bar in both Figs. 11A and 11B is 200 μm.

[0036] Fig. 12 depicts a quantification of *Axin2* expression levels by qPCR at 24 hours after start of explant culture in RSPO1 alone (control) or RSPO1 + WNT mimetic at 5nM. Expression for different conditions was normalized to *actinB* expression and relative to control (no WNT mimetic).

[0037] Fig. 13 depicts WNT receptor expression levels determined by *in situ* hybridization in naïve mouse lacrimal gland tissue. Optical micrograph histology images with probe signal in pink, scale bar is 100 μm.

[0038] Fig. 14 depicts WNT receptor expression levels determined by *in situ* hybridization in healthy human lacrimal gland tissue. Optical micrograph histology images with probe signal in pink, scale bar is 100 μm.

[0039] Fig. 15 depicts camera-based fluorescence microscope image showing four samples of naïve mouse lacrimal cells: a control (RSPO1 only) sample and three WNT mimetic samples after 14 days exposure (10 mpk, IP, two times a week) stained with 4′,6-diamidino-2-phenylindole (DAPI) and anti-Ki67, marker of proliferative cells (green).

[0040] Fig. 16 depicts a graph quantifying the relative weight (lacrimal gland weight/body weight) of the lacrimal gland following 14 days' treatment with various WNT mimetics. (WNT mimetic 3mpk, RSPO2 0.1 mpk, IP, twice a week). Figure legend is left to right in graph.

[0041] Fig. 17 depicts a schematic model of an *in vivo* mouse experiment wherein dry-eye disease was modeled by local injection of IL1a. Red represents the ipsilateral side injected with recombinant IL-1α and WNT mimetic (or control anti-GFP). Black represents the contralateral control side.

[0042] Figs. 18A and 18B depict graphs quantifying expression of WNT target genes *Axin2* (Fig. 18A) and *Rnf43* (Fig. 18B) after exposure to IL1a + WNT mimetic and IL1a + anti-GFP for 8 hours and 24 hours. Expression for different conditions was normalized to *actinB* expression and relative to control (8-hours exposure to GFP).

[0043] Fig. 19 depicts average tear volume secretion on the ipsilateral side over a five-day time course for four experimental conditions: (1) local administration of WNT mimetic (10 μg intra-lacrimal gland); (2) local administration of anti-GFP (10 μg intra-lacrimal gland); (3) systemic administration of WNT mimetic (200 μg IP); and (4) systemic administration of anti-GFP (200 μg IP).

[0044] Fig. 20 depicts a bar graph quantifying pathology scoring of atrophy/degeneration and of inflammation in mice 3 days following lacrimal gland injection with IL1a and four

different experimental treatment conditions: (1) local administration of WNT mimetic (10 μg intra-lacrimal gland); (2) local administration of anti-GFP (10 μg intra-lacrimal gland); (3) systemic administration of WNT mimetic (200 μg IP); and (4) systemic administration of anti-GFP (200 μg IP). Figure legend is shown left to right for each category.

[0045] Figs. 21A and 21B depict optical micrograph images of lacrimal gland sections stained with hematoxylin and eosin (H&E) stain from mice injected with IL1a + WNT mimetic and IL1a + anti-GFP. Scale bar in both images is 100 μm.

[0046] Fig. 22 shows salivary gland weight (in grams) after 14 days of biweekly treatment with WNT mimetics at 3 mg/kg. Figure legend is shown left to right in graph.

[0047] Fig. 23 shows salivary gland histology after two weeks of 3 mg/kg dosing of various WNT mimetics. Panel A represents images of salivary gland histology by HE staining per treatment group at day 14. Pan B shows staining for Ki67 (proliferation marker) in brown per treatment group at day 14. Scale bars 200 μm.

[0048] Figs. 24A and 24B show salivary gland histology after two week 10 mg/kg dosing. Fig. 24A shows representative images of salivary gland histology by HE staining per treatment group at day 14. Fig. 24B shows quantification of mucinous acini area (white) over serous acini area by Image J per treatment group. Scale bar 200 μm.

[0049] Fig. 25 is a graph representing the salivary gland weight (in grams) after two-week dosing with RSPO2-Fc at various concentrations. Figure legend is shown left to right in graph.

[0050] Figs. 26A and 26B show murine salivary gland organoid expansion. Fig. 26A provides brightfield images of salivary gland organoids expansion from primary tissue at day 7 treated with RSPO1 or RSPO1 + L-F12578. Scale bars 200 μm. Fig. 26B shows the dose dependent mouse salivary gland organoid expansion measured as cell viability at day 7 for WNT mimetics with different FZD specificities.

[0051] Fig. 27 shows murine salivary gland organoid WNT receptor profile. Gene expression levels of Fzd and Lrp genes in mouse submandibular gland organoids measured by quantitative PCR.

[0052] Figs. 28A and 28B show the effect of treatment on Lupus mouse salivary gland. Fig. 28A provides salivary gland weight (in grams) for control (MRL/MpJ) and lupus (MRL-lpr) mice treated with Anti-GFP or L-F12578 for two weeks. Fig. 28B shows images of salivary gland histology by HE staining per treatment group for lupus and control mice at day 14. Scale bars 200 μm.

[0053] Fig. 29 shows WNT target gene Axin2 expression 24h after local injection in IL-1α model. All treatment groups 10 μg injection. Data normalized to anti-GFP control group. Significant elevation for positive control L-F12578 and 1SH1-03.

[0054] Fig. 30 shows WNT target gene *Axin2* expression 24h after local injection in IL-1a model. All treatment groups except anti-GFP control at three different doses: 10, 50 or 150 μg injection. Data normalized to anti-GFP control group. Significant elevation all 1SH1-03 groups and the high dose 1SH1-26 groups.

[0055] Fig. 31 shows tear volume measurements using phenol red thread in the IL-1a model. Data represents an average of twelve animals. Significant tear volume elevation in several treatment groups on day 2 and 3 compared to Anti-GFP control. At day 2, the lines from top to bottom correspond to: L-F12578, 1SH1-03, 1SH1-36, 1SH1-26, and anti-GFP.

[0056] Fig. 32 illustrates WNT target gene Axin2 expression 3 days after local injection in IL-1α model. 1SH1-03 treatment groups at two different doses: 10 or 150 μg injection. Data normalized to anti-GFP control group. Significant elevation of Axin2 1SH1-03 high dose on day 3.

[0057] Fig. 33 shows tear volume measurements using phenol red thread in the IL-1α model. Data represents an average of twelve animals. Significant tear volume elevation in several treatment groups on day 2, 3, 4 compared to Anti-GFP control. At day 3, the lines from top to bottom are: L-F12578, 1SH1-36, 1SH1-03, 1SH1-26, and anti-GFP.

[0058] Figs. 34A and 34B show duct ligation model. Fig. 34A is a schematic representation of lacrimal gland duct ligation on the ipsilateral side (blue). Other side (contralateral, red) left as control. Fig. 34B is a study timeline of duct ligation model; 3-day closure and tear volume measurements and take down after ligation removal.

[0059] Figs. 35A and 35B show duct ligation damage. Fig. 35A shows tear volume measurements using phenol red thread after 3 days of duct closure. Ligation results in a strong decrease of tear volume that recovers over 2-3 weeks. At day 0, the top line is contralateral, and the bottom line is ipsilateral. Fig. 35B shows representative images of lacrimal gland histology following duct ligation. Severe atrophy on day 7 that slowly recovers on day 14 and day 21 compared to control.

[0060] Fig. 36 shows WNT target gene Axin2 expression at 24h in duct ligation model, treatment after 3-day duct closure. Treatment groups at two different doses: 10 or 100 μg injection. Data normalized to anti-GFP control group. Significant elevation of Axin2 1SH1-03 and positive control L-F12578.

[0061] Fig. 37 tear volume measurements for one week using phenol red thread after 3 days of duct closure. Significant elevation of tear volume on day 7 in the positive control L-F12578 and 100 µg of 1SH1-03. At day 7, the lines from top to bottom are: L-F12578 (10 ug), 1SH1-03 (100 ug), 1SH1-03 (10 ug), and anti-GFP.

[0062] Fig. 38 shows the quantification of proliferative acinar cells (Mist1+ Ki67+) in the ipsilateral and contralateral glands on day 7 of duct ligation study (from Fig. 34). Increase in proliferative cells in ipsilateral side and high dose 1SH1-03 contralateral side. For each set of bars, the bars from left to right are: GFP, L-12578 (10 ug), 1SH1-03 (10 ug), and 1SH1-03 (100 ug).

[0063] Fig. 39 shows tear volume measurements for two weeks using phenol red thread after 3 days of duct closure. Significant elevation of tear volume after day 7 in the positive control L-F12578 and 1SH1-03. At the last timepoint, the lines from top to bottom are: L-F12578 (10 ug), 1SH1-03 (100 ug), 1SH1-03 (10 ug), and anti-GFP.

[0064] Fig. 40 shows quantification of proliferative acinar cells (Mist1+ Ki67+) in the ipsilateral and contralateral glands on day 14 of duct ligation study (from Fig. 39). Increase in proliferative cells in ipsilateral side and high dose 1SH1-03 contralateral side. For each set of bars, the bars from left to right correspond to: GFP, G211-18R5, 1SH1-03 (10ug), and 1SH-03 (100 ug).

[0065] Figs. 41A and 41B show in vitro proliferation of acinar cells. Fig. 41A shows representative images of lacrimal gland cells treated with control or 10 nM 1SH1-03. WNT activation using 1SH1-03 results in larger organoids on day 7. Fig. 41B shows cell viability quantification of organoids on day 7 expanded in control medium or different doses of 1SH1-03. Dose-response effect with significantly more cells with 1SH1-03.

[0066] Fig. 42 shows the quantification of salivary gland proliferation in animals upon two-week systemic dosing in animals. Administration of L-F12578 has more proliferative epithelial cells (Ki67+/ECad+) on Day 7 compared to the control group and other timepoints. Vehicle is left, and L-F12578 is right for each timepoint. For each timepoint, the left bar is Vehicle, and the right bar is L-F12578.

[0067] Fig. 43 shows the quantification of salivary gland tissue weight in animals upon two-week systemic dosing in animals. The administration of L-F12578 significantly elevates organ weight at day 7, 9, 11 and 14 compared to control. Vehicle is left, and L-F12578 is right for each timepoint. For each timepoint, the left bar is Vehicle, and the right bar is L-F12578.

[0068] It will be readily apparent to persons having ordinary skill in the art that the above figures are illustrative only and do not limit the scope of the instant disclosure.

DETAILED DESCRIPTION

I. Definitions

[0069] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise.

[0070] All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

[0071] "Activity" of a molecule may describe or refer to the binding of the molecule to a ligand or to a receptor, to catalytic activity, to the ability to stimulate gene expression, to antigenic activity, to the modulation of activities of other molecules, and the like. "Activity" of a molecule may also refer to activity in modulating or maintaining cell-to-cell interactions, e.g., adhesion, or activity in maintaining a structure of a cell, e.g., cell membranes or cytoskeleton. "Activity" may also mean specific activity, e.g., [catalytic activity]/[mg protein], or [immunological activity]/[mg protein], or the like.

[0072] The terms "administering" or "introducing" or "providing", as used herein, refer to delivery of a composition to a cell, to cells, tissues and/or organs of a subject, or to a subject. Such administering or introducing may take place *in vivo*, *in vitro* or *ex vivo*.

[0073] As used herein, the term "antibody" means an isolated or recombinant binding agent that comprises the necessary variable region sequences to specifically bind an antigenic epitope. Therefore, an antibody is any form of antibody or fragment thereof that exhibits the desired biological activity, e.g., binding the specific target antigen. Thus, it is used in the broadest sense and specifically covers monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, human antibodies, humanized antibodies, chimeric antibodies, nanobodies, diabodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments including, but not limited to, scFv, Fab, and Fab2, so long as they exhibit the desired biological activity.

[0074] "Antibody fragments" comprise a portion of an intact antibody, for example, the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies (e.g., Zapata et al.,

Protein Eng. 8(10): 1057-1062 (1995)); single-chain antibody molecules (e.g., scFv); and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen combining sites and is still capable of cross-linking antigen.

[0075] The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and 30 additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. In certain embodiments, a binding agent (e.g., a WNT surrogate molecule or binding region thereof, or a WNT antagonist) is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules.

[0076] The term "antigen-binding fragment" as used herein refers to a polypeptide fragment that contains at least one complementarity-determining region (CDR) of an immunoglobulin heavy and/or light chain, or of a VHH/sdAb (single domain antibody) or Nanobody® (Nab), that binds to the antigen of interest, in particular to one or more Fzd receptors, or to LRP5 and/or LRP6. In this regard, an antigen-binding fragment of the herein described antibodies may comprise 1, 2, 3, 4, 5, or all 6 CDRs of a VH and VL from antibodies that bind one or more Fzd receptors or LRP5 and/or LRP6.

[0077] As used herein, the terms "biological activity" and "biologically active" refer to the activity attributed to a particular biological element in a cell. For example, the "biological activity" of a WNT agonist, or fragment or variant thereof refers to the ability to mimic or enhance WNT signals. As another example, the biological activity of a polypeptide or functional fragment or variant thereof refers to the ability of the polypeptide or functional fragment or variant thereof to carry out its native functions of, e.g., binding, enzymatic activity, etc. In some embodiments, a functional fragment or variant retains at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% of an activity of the corresponding native protein or nucleic acid. As a third example, the biological activity of a gene regulatory element, e.g., promoter, enhancer, Kozak sequence, and the like, refers to the ability of the regulatory element or functional fragment or variant thereof to regulate, i.e., promote, enhance, or activate the translation of, respectively, the expression of the gene to which it is operably linked.

[0078] The term "bifunctional antibody," as used herein, refers to an antibody that comprises a first arm having a specificity for one antigenic site and a second arm having a specificity for a different antigenic site, i.e., the bifunctional antibodies have a dual specificity.

[0079] "Bispecific antibody" is used herein to refer to a full-length antibody that is generated by quadroma technology (*see* Milstein et al., *Nature*, 305(5934): 537-540 (1983)), by chemical conjugation of two different monoclonal antibodies (*see* Staerz et al., *Nature*, 314(6012): 628-631 (1985)), or by knob-into-hole or similar approaches, which introduce mutations in the Fc region (*see* Holliger et al., *Proc. Natl. Acad. Sci. USA*, 90(14): 6444–6448 (1993)), resulting in multiple different immunoglobulin species of which only one is the functional bispecific antibody. A bispecific antibody binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). By this definition, a bispecific antibody has two distinct antigen-binding arms (in both specificity and CDR sequences) and is monovalent for each antigen to which it binds.

[0080] By "comprising," it is meant that the recited elements are required in, for example, the composition, method, kit, etc., but other elements may be included to form the, for example, composition, method, kit etc. within the scope of the claim. For example, an expression cassette "comprising" a gene encoding a therapeutic polypeptide operably linked to a promoter is an expression cassette that may include other elements in addition to the gene and promoter, e.g., poly-adenylation sequence, enhancer elements, other genes, linker domains, etc.

[0081] By "consisting essentially of," it is meant a limitation of the scope of the, for example, composition, method, kit, etc., described to the specified materials or steps that do not materially affect the basic and novel characteristic(s) of the, for example, composition, method, kit, etc. For example, an expression cassette "consisting essentially of" a gene encoding a therapeutic polypeptide operably linked to a promoter and a polyadenylation sequence may include additional sequences, e.g., linker sequences, so long as they do not materially affect the transcription or translation of the gene. As another example, a variant, or mutant, polypeptide fragment "consisting essentially of" a recited sequence has the amino acid sequence of the recited sequence plus or minus about 10 amino acid residues at the boundaries of the sequence based upon the full length naïve polypeptide from which it was derived, e.g., 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 residue less than the recited bounding amino acid

residue, or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues more than the recited bounding amino acid residue.

[0082] By "consisting of," it is meant the exclusion from the composition, method, or kit of any element, step, or ingredient not specified in the claim. For example, a polypeptide or polypeptide domain "consisting of" a recited sequence contains only the recited sequence.

[0083] A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter.

[0084] An "expression vector" is a vector, e.g., plasmid, minicircle, viral vector, liposome, and the like as discussed herein or as known in the art, comprising a region which encodes a gene product of interest, and is used for effecting the expression of the gene product in an intended target cell. An expression vector also comprises control elements, e.g., promoters, enhancers, untranslated regions (UTRs), miRNA targeting sequences, etc., operatively linked to the encoding region to facilitate expression of the gene product in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

[0085] As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid

sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

[0086] The terms "individual," "host," "subject," and "patient" are used interchangeably herein, and refer to a mammal, including, but not limited to, human and non-human primates, including simians and humans; mammalian sport animals (e.g., horses); mammalian farm animals (e.g., sheep, goats, etc.); mammalian pets (dogs, cats, etc.); and rodents (e.g., mice, rats, etc.).

[0087] "Humanized" antibodies or fragments thereof refers to antibodies or fragments thereof from non-human species whose protein sequences have been modified to increase their similarity to antibody variants produced naturally in humans. The process of "humanization" is usually applied to monoclonal antibodies developed for administration to humans.

[0088] A "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), single domain antibodies (sdAbs, also known as Nanobodies), variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen- binding fragment (epitope recognition site) of the required specificity and the ability to bind to an epitope, including WNT surrogate molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody".

[0089] The term "native" or "wild-type" as used herein refers to a nucleotide sequence, e.g., gene, or gene product, e.g., RNA or protein, that is present in a wild-type cell, tissue, organ, or organism. The term "variant" as used herein refers to a mutant of a reference polynucleotide or polypeptide sequence, for example a native polynucleotide or polypeptide sequence, i.e., having less than 100% sequence identity with the reference polynucleotide or polypeptide sequence. Put another way, a variant comprises at least one amino acid difference (e.g., amino acid substitution, amino acid insertion, amino acid deletion) relative to

a reference polynucleotide sequence, e.g., a native polynucleotide or polypeptide sequence. For example, a variant may be a polynucleotide having a sequence identity of 50% or more, 60% or more, or 70% or more with a full-length native polynucleotide sequence, e.g., an identity of 75% or 80% or more, such as 85%, 90%, or 95% or more, for example, 98% or 99% identity with the full-length native polynucleotide sequence. As another example, a variant may be a polypeptide having a sequence identity of 70% or more with a full-length native polypeptide sequence, e.g., an identity of 75% or 80% or more, such as 85%, 90%, or 95% or more, for example, 98% or 99% identity with the full-length native polypeptide sequence. Variants may also include variant fragments of a reference, e.g., native, sequence sharing a sequence identity of 70% or more with a fragment of the reference, e.g., native, sequence, e.g., an identity of 75% or 80% or more, such as 85%, 90%, or 95% or more, for example, 98% or 99% identity with the native sequence.

[0090] "Operatively linked" or "operably linked" refers to a juxtaposition of genetic elements, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained.

[0091] As used herein, the terms "polypeptide," "peptide," and "protein" refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, to include disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component.

[0092] The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double-stranded and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0093] A polynucleotide or polypeptide has a certain percent "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases

or amino acids are the same when comparing the two sequences. The terms "identical" or "identity" when used in the context of two or more nucleic acids or polypeptide sequences, refer to the number or percentage of residues that are the same in a sequence of interest and a reference sequence. The percentage can be calculated by optimally aligning the sequence of interest to the reference sequence; comparing the two sequences over the entire length of the reference sequence; determining the number of positions at which the identical amino acid residue or nucleic acid base occurs in both sequences to yield the number of matched positions; dividing the number of matched positions by the total number of positions in the reference sequence adjusted by adding the number of gap positions introduced into the reference sequence in generating the alignment; and multiplying the result by 100 to yield the percentage of sequence identity. When comparing DNA and RNA, thymine (T) and uracil (U) can be considered equivalent Sequence "identity" may be determined by using the stand-alone executable BLAST engine program for blasting two sequences (bl2seq), which can be retrieved from the National Center for Biotechnology Information (NCBI) ftp site or over the worldwide web at ncbi.nlm.nih.gov/BLAST/, using the default parameters (Tatusova and Madden, FEMS Microbiol Lett., 1999, 174, 247-250; which is incorporated herein by reference in its entirety).

[0094] A "promoter" as used herein encompasses a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis, i.e., a minimal sequence sufficient to direct transcription. Promoters and corresponding protein or polypeptide expression may be ubiquitous, meaning strongly active in a wide range of cells, tissues and species or cell-type specific, tissue-specific, or species specific. Promoters may be "constitutive," meaning continually active, or "inducible," meaning the promoter can be activated or deactivated by the presence or absence of biotic or abiotic factors. Also included in the nucleic acid constructs or vectors of the invention are enhancer sequences that may or may not be contiguous with the promoter sequence. Enhancer sequences influence promoter-dependent gene expression and may be located in the 5' or 3' regions of the native gene.

[0095] "Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature.

[0096] As used herein "Sjögren's syndrome" or "Sjogren's syndrome" is a chronic autoimmune disease affecting the lacrimal and salivary glands, defined according to customary international diagnostic criteria such as described in Vitali et al (2002)

Ann.Rheum. Dis. 61:554. Sjögren's syndrome is one of two major subclasses of aqueous-deficient dry-eye diseases, distinguished from non-Sjögren's syndrome type.

[0097] As used herein, "SWAP™" (Surrozen WNT-signal activating proteins) refers to WNT mimetic compounds comprising engineered bi-specific full-length immunoglobulin-G (IgG) antibodies that, like WNT proteins, directly activate the canonical WNT-signaling pathway in a target tissue, e.g., lacrimal gland tissue.

[0098] As used herein, "SWEETS™" (Surrozen WNT-signal enhancer engineered for tissue specificity) refers to antibody-based R-spondin mimetic compounds as described in US20200048324.

[0099] The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof, e.g., reducing the likelihood that the disease or symptom thereof occurs in the subject, and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal and includes: (a) inhibiting the disease, i.e., arresting or slowing its development; or (b) relieving the disease, i.e., causing regression or reduction of the disease or reducing the severity of the disease. The therapeutic agent may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to complete loss of function in the affected tissues. The subject therapy will desirably be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease.

[0100] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, molecular biology techniques), microbiology, biochemistry and immunology, which are within the scope of those of skill in the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D. M. Weir & C. C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J. M. Miller & M. P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in

Immunology" (J. E. Coligan et al., eds., 1991), each of which is expressly incorporated by reference herein.

[0101] Several aspects of the invention are described below with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of the invention. One having ordinary skill in the relevant art, however, will readily recognize that the invention can be practiced without one or more of the specific details or with other methods. The present invention is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with the present invention.

[0102] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having", "has", "with", or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term "comprising".

[0103] The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term "about" meaning within an acceptable error range for the particular value should be assumed.

[0104] All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0105] It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

[0106] Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art and the practice of the present invention will employ, conventional techniques of microbiology and recombinant DNA technology, which are within the knowledge of those of skill of the art.

II. General

[0107] The present invention provides methods of modulating WNT signals to treat lacrimal gland disorders due to, including but not limited to, Sjögren's syndrome disorder, chronic graft versus host disease (cGHVD), rheumatoid arthritis (RA), Stephen's Johnson syndrome, Ocular Rosacea, chemotherapy, radiation oncology treatments, diabetes, roasacea, lupus, etc.

[0108] WNT ("Wingless-related integration site" or "Wingless and Int-1" or "Wingless-Int") ligands and their signals play key roles in the control of development, homeostasis and regeneration of many essential organs and tissues, including bone, liver, skin, stomach, intestine, kidney, central nervous system, mammary gland, taste bud, ovary, cochlea, lung, and many other tissues (reviewed, e.g., by Clevers, Loh, and Nusse, 2014; 346:1248012). Modulation of WNT signaling pathways has potential for treatment of degenerative diseases and tissue injuries.

[0109] One of the challenges for modulating WNT signaling as a therapeutic is the existence of multiple WNT ligands and WNT receptors, Frizzled 1-10 (Fzd1-10), with many tissues expressing multiple and overlapping Fzds. Canonical WNT signals also involve Low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) or Low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) as co-receptors, which are broadly expressed in various tissues, in addition to Fzds. R-spondins 1–4 are a family of ligands that amplify WNT signals. Each of the R-spondins work through a receptor complex that contains Zinc and Ring Finger 3 (ZNRF3) or Ring Finger Protein 43 (RNF43) on one end and a Leucine-rich repeat-containing G-protein coupled receptor 4-6 (LGR4-6) on the other (reviewed, e.g., by Knight & Hankenson 2014, *Matrix Biol.*; 37: 157-161). R-spondins might also work through additional mechanisms of action. ZNRF3 and RNF43 are two membrane-bound E3 ligases specifically targeting WNT receptors (Fzd1-10 and LRP5 or LRP6) for degradation. Binding

of an R-spondin to ZNRF3/RNF43 and LGR4-6 causes clearance or sequestration of the ternary complex, which removes E3 ligases from WNT receptors and stabilizes WNT receptors, resulting in enhanced WNT signals. Each R-spondin contains two Furin domains (1 and 2), with Furin domain 1 binding to ZNRF3/RNF43, and Furin domain 2 binding to LGR4-6. Fragments of R-spondins containing Furin domains 1 and 2 are sufficient for amplifying WNT signaling. While R-spondin effects depend on WNT signals, since both LGR4-6 and ZNRF3/RNF43 are widely expressed in various tissues, the effects of R-spondins are not tissue-specific.

[0110] Activating WNT signaling by a WNT agonist may be used for the treatment of a variety of lacrimal gland diseases and disorders, including dry-eye diseases. Similarly, amplifying WNT signaling by RSPO or an RSPO mimetic may be used for the treatment of a variety of lacrimal gland diseases and disorders, including various dry eye and salivary gland diseases. A WNT agonist molecule may also be used for the treatment of dry eye and salivary gland disorders. In particular, active WNT signaling can provide a major stem cell maintenance signal and plays a key role in regulating regeneration of the acinar cells, e.g., in salivary glands.

III. Engineered WNT Agonists

[0111] The present disclosure provides engineered WNT agonists and contemplates the use of engineered WNT agonists to stimulate, agonize, or promote WNT signaling, e.g., through the canonical WNT/β-catenin signaling pathway. Such engineered WNT agonists may also be referred to as WNT/ β -catenin signaling agonists or Wnt mimetics.

[0112] The disclosure provides engineered Wnt mimetics with drug-like properties, particularly in the form of recombinant, bi-specific antibodies that bring together Fzd and Lrp to stimulate signaling, mimicking endogenous Wnt ligands. The Wnt mimetics of the disclosure may freely diffuse, access damaged tissues and guide tissue repair where Wnt signals are needed. The disclosure also provides Wnt mimetics that are capable of repairing damaged lacrimal or salivary gland tissue without being combined with RSPO.

[0113] In some embodiments, the WNT/β-catenin signaling antagonist or agonist can include binding agents or epitope binding domains that bind one or more Fzd receptors and inhibit or enhance WNT signaling. In certain embodiments, the agent or antibody specifically binds to the cysteine-rich domain (CRD) within the human frizzled receptor(s) to which it binds. Additionally, antagonistic binding agents containing epitope binding domains against LRP can also be used. In some embodiments, the WNT/β-catenin antagonist possesses

binding agents or epitope binding domains that bind E3 ligases ZNRF3/RNF43 and one or more FZD receptors or one or more LRP co-receptors to promote the degradation of FZD or LRP receptors, and this molecule can also contain a binding domain that binds a cell type specific epitope for targeting. The E3 ligase agonist antibodies or fragments thereof can be single molecules or combined with other WNT antagonists, e.g., Fzd receptor antagonists, LRP receptor antagonists, etc.

[0114] As is well known in the art, an antibody is an immunoglobulin molecule capable of specific binding to a target such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least on epitope binding domain, located on the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also fragments thereof containing epitope binding domains (e.g., dAb, Fab, Fab', $(F(ab')_2$, Fv, single chain (scFv), VHH (i.e., Nanobodies®) or single domain antibodies (sdAb), DVD-Igs (also known as Fv-Igs), synthetic variants thereof, naturally occurring variants, fusion proteins comprising and epitope binding domain, humanized antibodies, chimeric antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen-binding site or fragment (epitope recognition site) of the required specificity. "Diabodies," multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al., *Proc. Natl. Acad. Sci. USA* 90 6444-6448, 1993) are also a particular form of antibody contemplated herein. Minibodies comprising a scFv joined to a CH3 domain are also included herein (S. Hu et al., *Cancer Res.*, 56, 3055-3061, 1996). *See e.g.*, Ward, E. S. et al., *Nature* 341, 544-546 (1989); Bird et al., *Science*, 242, 423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA*, 85, 5879-5883, 1988); PCT/US92/09965; WO94/13804; P. Holliger et al., *Proc. Natl. Acad. Sci. USA* 90 6444–6448, 1993; Y. Reiter et al., *Nature Biotech*, 14, 1239-1245, 1996; S. Hu et al., *Cancer Res.*, 56, 3055-3061, 1996; C. Bever et al., *Anal Bioanal Chem.* 2016 Sept; 408(22); 5985–6002.

[0115] The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the F(ab')2 fragment which comprises both antigen-binding sites. An Fv fragment for use according to certain embodiments of the present disclosure can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions of an IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes

a non-covalent VH::VL heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar et al. (1972) *Proc. Nat. Acad. Sci. USA* 69:2659-2662; Hochman et al. (1976) *Biochem* 15:2706-2710; and Ehrlich et al. (1980) *Biochem* 19:4091-4096.

[0116] In certain embodiments, single chain Fv or scFV antibodies are contemplated. For example, Kappa bodies (Ill et al., *Prot. Eng.* 10: 949-57 (1997)); minibodies (Martin et al., *EMBO J* 13: 5305-9 (1994)); diabodies (Holliger et al., *Proc. Nat. Acad. Sci.* 90: 6444-8 (1993)); or Janusins (Traunecker et al., *EMBO J* 10: 3655-59 (1991) and Traunecker et al., *Int. J. Cancer Suppl.* 7: 51-52 (1992)), may be prepared using standard molecular biology techniques following the teachings of the present application with regard to selecting antibodies having the desired specificity. In still other embodiments, bispecific or chimeric antibodies may be made that encompass the ligands of the present disclosure. For example, a chimeric antibody may comprise CDRs and framework regions from different antibodies, while bispecific antibodies may be generated that bind specifically to one or more Fzd receptors through one binding domain and to a second molecule through a second binding domain. These antibodies may be produced through recombinant molecular biological techniques or may be physically conjugated together.

[0117] A single chain Fv (scFv) polypeptide is a covalently linked VH:VL heterodimer which is expressed from a gene fusion including VH- and VL-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) *Proc. Nat. Acad. Sci. USA* 85(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated—but chemically separated—light and heavy polypeptide chains from an antibody V region into an scFv molecule which will fold into a three-dimensional structure substantially similar to the structure of an antigen-binding site. *See, e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston et al.; and U.S. Pat. No. 4,946,778, to Ladner et al.

[0118] In certain embodiments, an antibody as described herein is in the form of a diabody. Diabodies are multimers of polypeptides, each polypeptide comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (e.g., by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

[0119] A dAb fragment of an antibody consists of a VH domain (Ward, E. S. et al., (1989) *Nature* 341:544-546).

[0120] Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. & Winter G., *Curr. Opin. Biotech.* 4, 446-449 (1993)), e.g., prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

[0121] Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in E. coli. Diabodies (and many other polypeptides such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by knobs-into-holes engineering (J. B. B. Ridgeway et al., *Protein Eng.*, 9, 616-621 (1996)).

[0122] In certain embodiments, the antibodies described herein may be provided in the form of a UniBody®. A UniBody® is an IgG4 antibody with the hinge region removed (see GenMab Utrecht, The Netherlands; see also, e.g., US2009/0226421). This proprietary antibody technology creates a stable, smaller antibody format with an anticipated longer therapeutic window than current small antibody formats. IgG4 antibodies are considered inert and thus do not interact with the immune system. Fully human IgG4 antibodies may be modified by eliminating the hinge region of the antibody to obtain half-molecule fragments having distinct stability properties relative to the corresponding intact IgG4 (GenMab, Utrecht). Halving the IgG4 molecule leaves only one area on the UniBody® that can bind to cognate antigens (e.g., disease targets) and the UniBody® therefore binds univalently to only one site on target cells.

[0123] In certain embodiments, antibodies and antigen-binding fragments thereof as described herein include a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain framework region (FR) set which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light chain, these regions aredenoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site,

therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (e.g., a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

[0124] As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures—regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

[0125] A "monoclonal antibody" refers to a homogeneous antibody population wherein the monoclonal antibody is comprised of amino acids (naturally occurring and non-naturally occurring) that are involved in the selective binding of an epitope. Monoclonal antibodies are highly specific, being directed against a single epitope. The term "monoclonal antibody" encompasses not only intact monoclonal antibodies and full-length monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')2, Fv), single chain (scFv), Nanobodies®, variants thereof, fusion proteins comprising an antigen-binding fragment of a monoclonal antibody, humanized monoclonal antibodies, chimeric monoclonal antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen- binding fragment (epitope recognition site) of the required specificity and the ability to bind to an epitope, including WNT surrogate molecules disclosed herein. It is not intended to be limited as regards the source of the antibody or the manner in which it is made (e.g., by hybridoma, phage selection, recombinant expression, transgenic animals, etc.). The term includes whole immunoglobulins as well as the fragments etc. described above under the definition of "antibody".

[0126] In certain embodiments, the antibodies of the present disclosure may take the form of a single domain antibody (sdAb). SdAb technology was originally developed following the discovery and identification that camelidae (e.g., camels, alpacas, and llamas) possess fully functional antibodies that consist of heavy chains only and therefore lack light chains. These heavy-chain only antibodies contain a single variable domain (VHH) and two constant domains (CH2, CH3). The cloned and isolated single variable domains have full antigen binding capacity and are very stable. These single variable domains, with their unique structural and functional properties, form the basis of sdAbs. SdAb are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts, e.g., E. coli (see, e.g., U.S. Pat. No. 6,765,087), molds (for example Aspergillus or Trichoderma) and yeast (for example Saccharomyces, Kluyvermyces, Hansenula, or Pichia (see, e.g., U.S. Pat. No. 6,838,254). The production process is scalable and multi-kilogram quantities of sdAbs have been produced. SdAbs may be formulated as a ready-to-use solution having a long shelf life. The Nanoclone® method (see, e.g., WO 06/079372) is a proprietary method for generating sdAbs against a desired target, based on automated high-throughput selection of B-cells. SdAb are single-domain antigen-binding fragments of camelid-specific heavy-chain only antibodies. sdAb, also referred to as VHH antibodies, typically have a small size of around 15 kDa. *See* C. Bever et al., *Anal Bioanal Chem.* 2016 Sept; 408(22); 5985–6002.

[0127] Another antibody fragment contemplated is a dual-variable domain-immunoglobulin (DVD-Ig or Fv-Ig) is an engineered protein that combines the function and specificity of two monoclonal antibodies in one molecular entity. An Fv-Igis designed as an IgG-like molecule, except that each light chain and heavy chain contains two variable domains in tandem through a short peptide linkage, instead of one variable domain in IgG. The fusion orientation of the two variable domains and the choice of linker sequence are critical to functional activity and efficient expression of the molecule. An Fv-Ig can be produced by conventional mammalian expression systems as a single species for manufacturing and purification. An Fv-Ig has the specificity of the parental antibodies, is stable in vivo, and exhibits IgG-like physicochemical and pharmacokinetic properties. Fv-Igs and methods for making them are described in Wu, C., et al., *Nat Biotech*, 25:1290–1297 (2007)).

[0128] In certain embodiments, the antibodies or antigen-binding fragments thereof as disclosed herein are humanized. This refers to a chimeric molecule, generally prepared using recombinant techniques, having an antigen- binding site derived from an immunoglobulin from a non-human species and the remaining immunoglobulin structure of the molecule

based upon the structure and/or sequence of a human immunoglobulin. The antigen-binding site may comprise either complete variable domains fused onto constant domains or only the CDRs grafted onto appropriate framework regions in the variable domains. Epitope binding sites may be wild type or modified by one or more amino acid substitutions. This eliminates the constant region as an immunogen in human individuals, but the possibility of an immune response to the foreign variable region remains (LoBuglio, A. F. et al., (1989) *Proc Natl Acad Sci USA* 86:4220–4224; Queen et al., *PNAS* (1988) 86:10029–10033; Riechmann et al., *Nature* (1988) 332:323–327). Illustrative methods for humanization of the anti-Fzd or LRP antibodies disclosed herein include the methods described in U.S. Pat. No. 7,462,697.

[0129] Another approach focuses not only on providing human-derived constant regions but modifying the variable regions as well so as to reshape them as closely as possible to human form. It is known that the variable regions of both heavy and light chains contain three complementarity-determining regions (CDRs) which vary in response to the epitopes in question and determine binding capability, flanked by four framework regions (FRs) which are relatively conserved in a given species and which putatively provide a scaffolding for the CDRs. When nonhuman antibodies are prepared with respect to a particular epitope, the variable regions can be "reshaped" or "humanized" by grafting CDRs derived from nonhuman antibody on the FRs present in the human antibody to be modified. Application of this approach to various antibodies has been reported by Sato, K., et al., (1993) *Cancer Res* 53:851-856; Riechmann, L., et al., (1988) *Nature* 332:323-327; Verhoeyen, M., et al., (1988) *Science* 239:1534-1536; Kettleborough, C. A., et al., (1991) *Protein Engineering* 4:773-3783; Maeda, H., et al., (1991) Human Antibodies Hybridoma 2:124-134; Gorman, S. D., et al., (1991) *Proc Natl Acad Sci USA* 88:4181-4185; Tempest, P. R., et al., (1991) *Bio/Technology* 9:266-271; Co, M. S., et al., (1991) *Proc Natl Acad Sci USA* 88:2869-2873; Carter, P., et al., (1992) *Proc Natl Acad Sci USA* 89:4285-4289; and Co, M. S. et al., (1992) *J Immunol* 148:1149-1154. In some embodiments, humanized antibodies preserve all CDR sequences (for example, a humanized mouse antibody which contains all six CDRs from the mouse antibodies). In other embodiments, humanized antibodies have one or more CDRs (one, two, three, four, five, six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs from the original antibody.

[0130] In certain embodiments, the antibodies of the present disclosure may be chimeric antibodies. In this regard, a chimeric antibody is comprised of an antigen-binding fragment of an antibody operably linked or otherwise fused to a heterologous Fc portion of a different antibody. In certain embodiments, the heterologous Fc domain is of human origin. In other

embodiments, the heterologous Fc domain may be from a different Ig class from the parent antibody, including IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG (including subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. In further embodiments, the heterologous Fc domain may be comprised of CH2 and CH3 domains from one or more of the different Ig classes. As noted above with regard to humanized antibodies, the antigen-binding fragment of a chimeric antibody may comprise only one or more of the CDRs of the antibodies described herein (e.g., 1, 2, 3, 4, 5, or 6 CDRs of the antibodies described herein), or may comprise an entire variable domain (VL, VH or both).

[0131] The structures and locations of immunoglobulin CDRs and variable domains may be determined by reference to Kabat, E. A. et al., SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, 4th Edition, US Department of Health and Human Services. 1987, and updates thereof, now available on the Internet (immuno.bme.nwu.edu).

[0132] In certain embodiments, the antagonist or agonist binding agent binds with a dissociation constant (K_D) of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, or about 10 nM or less. For example, in certain embodiments, a FZD binding agent or antibody described herein that binds to more than one FZD, binds to those FZDs with a K_D of about 100nM or less, about 20 nM or less, or about 10 nM or less. In certain embodiments, the binding agent binds to one or more its target antigen with an EC50 of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, or about 1 nM 20 or less.

[0133] The antibodies or other agents of the present invention can be assayed for specific binding by any method known in the art. The immunoassays which can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as biolayer interferometry (BLI) analysis, FACS analysis, immunofluorescence, immunocytochemistry, Western blots, radioimmunoassays, ELISA, "sandwich" immunoassays, immunoprecipitation assays, precipitation reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays. Such assays are routine and well known in the art (*see, e.g.*, Ausubel et al, eds, 1994, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety).

[0134] For example, the specific binding of an antibody to a target antigen may be determined using ELISA. An ELISA assay comprises preparing antigen, coating wells of a 96 well microtiter plate with antigen, adding the antibody or other binding agent conjugated

to a detectable compound such as an enzymatic substrate (e.g., horse-radish peroxidase or alkaline phosphatase) to the well, incubating for a period of time and detecting the presence of the antigen. In some embodiments, the antibody or agent is not conjugated to a detectable compound, but instead a second conjugated antibody that recognizes the first antibody or agent is added to the well. In some embodiments, instead of coating the well with the antigen, the antibody or agent can be coated to the well and a second antibody conjugated to a detectable compound can be added following the addition of the antigen to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art (*see, e.g.*, Ausubel et al, eds, 1994, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1).

[0135] The binding affinity of an antibody or other agent to a target antigen and the off-rate of the antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (e.g., Fzd, LRP), or fragment or variant thereof, with the antibody of interest in the presence of increasing amounts of unlabeled antigen followed by the detection of the antibody bound to the labeled antigen. The affinity of the antibody and the binding off-rates can be determined from the data by scattered plot analysis. In some embodiments, BLI analysis is used to determine the binding on and off rates of antibodies or agents. BLI kinetic analysis comprises analyzing the binding and dissociation of antibodies from chips with immobilized antigens on their surface.

IV. Compositions of Methods of Use of Wnt Agonists

[0136] The present description provides methods of treating lacrimal gland diseases and disorders, including but not limited to dry-eye disorders, e.g., by activation and/or regeneration of lacrimal gland acinar cells, and compositions therefor.

[0137] It will be readily understood to persons skilled in the art that lacrimal gland is homologous to Meibomian gland and accessory lacrimal glands, and the methods and compositions described herein may be applied for treatment of the Meibomian glands and/or accessory lacrimal glands in aqueous-deficient or evaporative dry eye. Meibomian glands are oil glands which line the margin of eyelids. These glands cause the eyelids to secrete oils that blend with natural tears, preventing excessive evaporation of the natural tears. Patients suffering from ocular rosacea often have Meibomian gland dysfunction (MGD) where the eyelids secrete fewer oils resulting dry eyes. MGD is thought to be the leading cause of dry

eye disease. A WNT agonist molecule may also be used for the treatment of Meibomian gland disorders. In particular, active WNT signaling can potentially provide a maintenance signal to basal progenitor cells and plays a key role in regulating regeneration of the meibocytes (see, e.g., Parfitt et al (2016) *Stem Cell Rep.*7:399-410). In one aspect, the present invention comprises a method of regenerating lacrimal gland acinar cells in a subject, comprising administering a WNT signaling modulator to the subject. In an embodiment, the WNT signaling modulator may be an engineered WNT signaling modulator such as a SWAP™ compound. In another embodiment, the WNT signaling modulator is an engineered WNT agonist or an engineered WNT antagonist. In yet a further embodiment, the WNT signaling modulator is a tissue-specific WNT signal enhancing molecule, such as a SWEETS™ molecule. A WNT signaling modulator may also be a combination of a WNT agonist and a tissue-specific WNT signal enhancer.

[0138] In particular embodiments of any of the methods disclosed herein, the WNT agonist is selected from those disclosed in any of the following: PCT Application Publication No. WO 2016/040895; US Application Publication No. US 2017-0306029; US Application Publication No. US 2017-0349659; PCT Application Publication No. WO 2019/126398; PCT Application Publication No. WO 2020/01030, PCT Application Publication No. WO 2021/173726, or U.S. Application No. 17/806,624, all of which are herein incorporated by reference in their entireties. In particular embodiments of any of the methods disclosed herein, the tissue-specific WNT signal enhancing molecule is selected from those disclosed in any of the following: PCT Application Publication No. WO 2018/140821; US Application Publication No. US 2020-0048324; or PCT Application Publication No. WO 2020/14271, all of which are herein incorporated by reference in their entireties.

[0139] In an embodiment of the method of regenerating lacrimal gland acinar cells, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. In any embodiment, the engineered WNT agonist may be selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic. The WNT mimetic may be a SWAP™ compound. The R-spondin mimetic may be a SWEETS™ compound. The WNT mimetics may comprise one or more polypeptides comprising or having a polypeptide sequence set forth in any of SEQ ID Nos: 1-14 isoforms and homologs thereof, and suitable expression vectors therefor. The WNT mimetics may comprise one or more polypeptides having 80% to 100% homology with any of the polypeptide sequences set forth in SEQ ID Nos 1-14. In certain embodiments, the WNT mimetic comprises two polypeptide sequences having 80% to 100% homology with

any of the polypeptide sequences set forth in SEQ ID Nos 1-14. In certain embodiments, the WNT mimetic comprises two heavy chain and two light chain polypeptide sequences, each having 80% to 100% homology with any of the polypeptide sequences set forth in SEQ ID Nos 1-14. In particular embodiments, the heavy chain and light chain polypeptides present in the WNT mimetic have at least 80%, at least 90%, at least 95%, at least 98%, or 100% identity to any of following combinations: SEQ ID NOs 1 and 2; SEQ ID NOs: 3 and 4; SEQ ID NOs: 5 and 6; SEQ ID NOs: 7 and 8; or SEQ ID NOs: 9 and 10, or SEQ ID NOs: 11 and 14, or SEQ ID NOs: 12 and 14, or SEQ ID NOs: 13 and 14. In particular embodiments, the WNT mimetic has an IgG antibody structure comprising two heavy and two light chains, wherein the two heavy chains are bound to each other, and each of the light chains is bound to a different one of the two heavy chains.

[0140] In an embodiment of the method of regenerating lacrimal gland acinar cells, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8, and Lrp6, and/or Lrp5. For example, the WNT signaling modulator may target any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; or any one or more of the group consisting of: Fzd5 and Fzd8, while also targeting Lrp6 and/or Lrp5. For another example, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, and Fzd7.

[0141] In one embodiment of the method of regenerating lacrimal gland acinar cells, the method further comprises the step of administering at least one of the group consisting of: RSPO2, RSPO2 fragment, and engineered RSPO2 mimetic.

[0142] In some embodiment the WNT signaling modulator may be a superagonist of WNT platform. This superagonist activity was observed with LRP, FZD, and RSPO fused WNT molecule acting as a robust activator of WNT signaling pathway. the WNT superagonist is selected from those disclosed in following: PCT Application Publication No. WO 2021/173726.

[0143] In any embodiment, the WNT signaling modulator may be in a concentration ≥1nM. For example, the WNT signaling modulator may be in a concentration of 1nM, 1.1nM, 1.5nM, 2.0nM, 2.5nM, 3.0nM, 3.5nM, 4.0nM, 4.5nM, 5.0nM. In certain embodiments, the concentration of WNT signaling modulator may be ≥5nM. In any embodiment, the WNT signaling modulator may be administered in a therapeutically effective amount.

[0144] In any embodiment, the subject may be a live mammal. For example, the subject may be mouse, rat, dog, cat, horse, or cow. In any embodiment, the subject may be a human patient.

[0145] In any embodiment, the WNT signaling modulator may be administered systemically or locally. For example, the WNT signaling modulator may be administered locally via aqueous eyedrop solution or local intra-lacrimal gland injection.

[0146] In another aspect, the present invention comprises a method of treating a lacrimal gland disorder in a subject, comprising administering a WNT signaling modulator to the subject. In an embodiment, the WNT signaling modulator may be an engineered WNT signaling modulator. In another embodiment, the WNT signaling modulator is an engineered WNT agonist or an engineered WNT antagonist.

[0147] In an embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. In any embodiment, the engineered WNT agonist may be selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic. The WNT mimetic may be a SWAP™ compound. The R-spondin mimetic may be a SWEETS™ compound.

[0148] In an embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8, and Lrp6 and/or Lrp5. For example, the WNT signaling modulator may target any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; or any one or more of the group consisting of: Fzd5 and Fzd8, while also targeting Lrp6 and/or Lrp5. For another example, the WNT signaling modulator may affect expression of any one or more of: Fzd1, Fzd2, and Fzd7.

[0149] In one embodiment of the method of treating a lacrimal gland disorder, the method further comprises the step of administering at least one of the group consisting of: RSPO2, RSPO2 fragment, and engineered RSPO2 mimetic.

[0150] In any embodiment of the method of treating a lacrimal gland disorder, the WNT signaling modulator may be in a concentration ≥1nM. For example, the WNT signaling modulator may be in a concentration of 1nM, 1.1nM, 1.5nM, 2.0nM, 2.5nM, 3.0nM, 3.5nM, 4.0nM, 4.5nM, 5.0nM. In certain embodiments, the concentration of WNT signaling modulator may be ≥5nM. In any embodiment, the WNT signaling modulator may be administered in a therapeutically effective amount.

[0151] In any embodiment, the subject may be a live mammal. For example, the subject may be mouse, rat, dog, cat, horse, or cow. In any embodiment, the subject may be a human patient.

[0152] In any embodiment, the WNT signaling modulator may be administered systemically or locally. For example, the WNT signaling modulator may be administered locally via aqueous eyedrop solution or local intra-lacrimal gland injection.

[0153] In still another aspect, the present invention comprises a composition for the treatment of a dry-eye disorder in a subject, the composition comprising a WNT signaling modulator. In any embodiment of the composition, the dry-eye disorder may be a Sjögren's syndrome disorder.

[0154] In an embodiment of the composition, the WNT signaling modulator may comprise at least one engineered bi-specific full-length IgG antibody that directly activates a canonical WNT signaling pathway. The at least one engineered bi-specific full-length IgG antibody may be specific for any one or more of Fzd1, Fzd,2, Fzd5, Fzd7, Fzd8, and Lrp6 or Lrp5.

[0155] In an embodiment, the composition may further comprise an at least one additional agent, including an anti-inflammatory agent, an artificial tear agent, or an lacrimal gland secretagogue. In particular, the anti-inflammatory agent may be antibiotics or steroids including cyclosporine A (e.g., Restasis®) and lifitegrast ophthalmic solution (e.g., Xiidra®). Artificial tears agent may include non-prescription eye drops that simulate tears, e.g., hydroxypropyl cellulose (Lacrisert®) inserts. Lacrimal gland secretagogue agents may include varenicline nasal spray, which selectively agonizes nicotinic acetylcholine receptors. Also, contemplated are autologous blood serum eye drops.

[0156] In any embodiment of the composition, the composition may comprise therapeutically effective amounts of each of its components.

[0157] In any embodiment of the composition, the subject may be a live mammal. For example, the subject may be mouse, rat, dog, cat, horse, or cow. In any embodiment of the composition, the subject may be a human patient.

[0158] In further embodiments, pharmaceutical compositions comprising an expression vector, e.g., a viral vector, comprising a polynucleotide comprising a nucleic acid sequence encoding a WNT antagonist/agonist molecule described herein and one or more pharmaceutically acceptable diluent, carrier, or excipient are also disclosed. In certain embodiments, the nucleic acid sequence encoding the WNT antagonist molecule and the

nucleic acid sequence encoding the WNT agonist are in the same polynucleotide, e.g., expression cassette.

[0159] The present disclosure further contemplates a pharmaceutical composition comprising a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid encoding a WNT antagonist/agonist molecule and one or more pharmaceutically acceptable diluent, carrier, or excipient. In particular embodiments, the pharmaceutical composition further comprises a cell comprising an expression vector comprising a polynucleotide comprising a promoter operatively linked to a nucleic acid sequence encoding a WNT antagonist and a WNT agonist. In certain embodiments, the nucleic acid sequence encoding the WNT antagonist molecule and the nucleic acid sequence encoding the WNT agonist molecule are present in the same polynucleotide, e.g., expression cassette and/or in the same cell. In particular embodiments, the cell is a heterologous cell or an autologous cell obtained from the subject to be treated.

[0160] In particular embodiments, the cell is a stem cell, e.g., an adipose-derived stem cell or a hematopoietic stem cell. The present disclosure contemplates pharmaceutical compositions comprising a first molecule for delivery of a WNT antagonist molecule as a first active agent, and a WNT agonist as a second molecule. The first and second molecule may be the same type of molecule or different types of molecules. For example, in certain embodiments, the first and second molecule may each be independently selected from the following types of molecules: polypeptides, small organic molecules, nucleic acids encoding the first or second active agent (optionally DNA or mRNA, optionally modified RNA), vectors comprising a nucleic acid sequence encoding the first or second active agent (optionally expression vectors or viral vectors), and cells comprising a nucleic acid sequence encoding the first or second active agent (optionally an expression cassette).

[0161] The subject molecules, alone or in combination, can be combined with pharmaceutically acceptable carriers, diluents, excipients and reagents useful in preparing a formulation that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for mammalian, e.g., human or primate, use. Such excipients can be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. Examples of such carriers, diluents and excipients include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Supplementary active compounds can also be incorporated into the formulations. Solutions or suspensions used for the formulations can include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such

as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates; detergents such as Tween 20 to prevent aggregation; and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. In particular embodiments, the pharmaceutical compositions are sterile.

[0162] Pharmaceutical compositions may further include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, or phosphate buffered saline (PBS). In some cases, the composition is sterile and should be fluid such that it can be drawn into a syringe or delivered to a subject from a syringe. In certain embodiments, it is stable under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be, e.g., a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the internal compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0163] Sterile solutions can be prepared by incorporating the WNT antagonist/agonist antibody or antigen-binding fragment thereof (or encoding polynucleotide or cell comprising the same) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile- filtered solution thereof.

[0164] In one embodiment, the pharmaceutical compositions are prepared with carriers that will protect the antibody or antigen-binding fragment thereof against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art.

[0165] It may be advantageous to formulate the pharmaceutical compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active antibody or antigen-binding fragment thereof calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms is dictated by and directly dependent on the unique characteristics of the antibody or antigen-binding fragment thereof and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active antibody or antigen-binding fragment thereof for the treatment of individuals.

[0166] The pharmaceutical compositions can be included in a container, pack, or dispenser, e.g., eye dropper, e.g., a prefilled eye dropper, together with instructions for administration.

[0167] The pharmaceutical compositions of the present disclosure may be delivered to a subject in the form of a pill, capsule, cream, salve, syrup, dermal patch, suppository, intravenous drip, local injection aqueous solution, non-aqueous solution, eye wash solution, or any combination of thereof.

[0168] The pharmaceutical compositions of the present disclosure may be delivered to a subject by direct ophthalmic application, muscular injection, intra-lacrimal gland injection, subconjunctival injection, meibomian gland injection, intravenous injection, peritoneal injection, nasally, orally, rectally, or any combination thereof.

[0169] The pharmaceutical compositions of the present disclosure encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal comprising a human, is capable of providing (directly or indirectly) the biologically active antibody or antigen-binding fragment thereof.

[0170] The present disclosure includes pharmaceutically acceptable salts of a WNT antagonist/agonist molecule described herein. The term "pharmaceutically acceptable salt" refers to physiologically and pharmaceutically acceptable salts of the compounds of the present disclosure: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. A variety of pharmaceutically acceptable salts are known in the art and described, e.g., in "Remington's Pharmaceutical Sciences", 17th edition, Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, USA, 1985 (and more recent editions thereof), in the "Encyclopedia of Pharmaceutical Technology", 3rd edition, James Swarbrick (Ed.), Informa Healthcare USA (Inc.), NY, USA, 2007, and in *J. Pharm. Sci.* 66:2 (1977). Also, for a review on suitable salts, see "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, 2002). Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines.

[0171] Metals used as cations comprise sodium, potassium, magnesium, calcium, and the like. Amines comprise N-N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N- methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. Pharma Sci.*, 1977, 66, 119). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present disclosure.

[0172] In some embodiments, the pharmaceutical composition provided herein comprise a therapeutically effective amount of a WNT antagonist/agonist molecule or pharmaceutically acceptable salt thereof in admixture with a pharmaceutically acceptable carrier, diluent and/or excipient, for example saline, phosphate buffered saline, phosphate and amino acids, polymers, polyols, sugar, buffers, preservatives and other proteins. Exemplary amino acids, polymers and sugars and the like are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds, polyoxyethylene sorbitan fatty acid esters, sucrose, fructose, dextrose, maltose, glucose, mannitol, dextran, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, bovine or human serum albumin, citrate, acetate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine,

polyvinylpyrrolidone, polyethylene and glycol. Preferably, this formulation is stable for at least six months at 4°C.

[0173] In some embodiments, the pharmaceutical composition provided herein comprises a buffer, such as phosphate buffered saline (PBS) or sodium phosphate/sodium sulfate, tris buffer, glycine buffer, sterile water and other buffers known to the ordinarily skilled artisan such as those described by Good et al. (1966) *Biochemistry* 5:467. The pH of the buffer may be in the range of 6.5 to 7.75, preferably 7 to 7.5, and most preferably 7.2 to 7.4.

[0174] From the foregoing it will be appreciated that, although specific embodiments of the present disclosure have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the present disclosure. Accordingly, the present disclosure is not limited except as by the appended claims.

[0175] The broad scope of this invention is best understood with reference to the following example, which is not intended to limit the inventions to the specific embodiments.

EXAMPLES

I. Methods.

[0176] Standard methods in molecular biology were used, including methods described, e.g., in Maniatis et al. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Sambrook and Russell (2001) *Molecular Cloning*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, Calif. Standard methods also appear in Ausbel et al. (2001) *Current Protocols in Molecular Biology, Vols.* 1-4, John Wiley and Sons, Inc. New York, N.Y., which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

[0177] Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described, e.g., in Coligan et al. (2000) *Current Protocols in Protein Science, Vol.* 1, John Wiley and Sons, Inc., New York. Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described in, e.g., Coligan et al. (2000) *Current Protocols in Protein Science, Vol.* 2, John Wiley and Sons, Inc., New York; Ausubel et al. (2001) *Current Protocols in Molecular Biology, Vol.* 3, John Wiley and Sons, Inc., NY, N.Y.,

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pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, Mo.; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391. Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described, e.g., in Coligan et al. (2001) *Current Protocols in Immunology, Vol.* 1, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Harlow and Lane, supra. Standard techniques for characterizing ligand/receptor interactions are available in, e.g., Coligan et al. (2001) *Current Protocols in Immunology, Vol.* 4, John Wiley, Inc., New York.

[0178] Methods for flow cytometry, including fluorescence activated cell sorting detection systems (FACS®), are available in, e.g., Owens et al. (1994) *Flow Cytometry Principles for Clinical Laboratory Practice*, John Wiley and Sons, Hoboken, N.J.; Givan (2001) *Flow Cytometry*, 2nd ed.; Wiley-Liss, Hoboken, N.J.; Shapiro (2003) *Practical Flow Cytometry*, John Wiley and Sons, Hoboken, N.J. Fluorescent reagents suitable for modifying nucleic acids, including nucleic acid primers and probes, polypeptides, and antibodies, for use, e.g., as diagnostic reagents, are available In, e.g., Molecular Probes (2003) *Catalogue*, Molecular Probes, Inc., Eugene, Oreg.; Sigma-Aldrich (2003) *Catalogue*, St. Louis, Mo.

[0179] Standard methods of histology of the immune system are described in, e.g., Muller-Harmelink (ed.) (1986) *Human Thymus: Histopathology and Pathology*, Springer Verlag, New York, N.Y.; Hiatt, et al. (2000) *Color Atlas of Histology*, Lippincott, Williams, and Wilkins, Phila, Pa.; Louis, et al. (2002) *Basic Histology: Text and Atlas*, McGraw-Hill, New York, N.Y.

[0180] Software packages and databases for determining, e.g., antigenic fragments, leader sequences, protein folding, functional domains, glycosylation sites, and sequence alignments, are available, in, e.g., GenBank, Vector NTI® Suite (Informax, Inc, Bethesda, Md.); GCG Wisconsin Package (Accelrys, Inc., San Diego, Calif.); DeCypher® (TimeLogic Corp., Crystal Bay, Nev.); Menne et al. (2000) *Bioinformatics* 16: 741-742; Menne et al. (2000) *Bioinformatics Applications Note* 16:741-742; Wren et al. (2002) *Comput. Methods Programs Biomed.* 68:177-181; von Heijne (1983) *Eur. J. Biochem.* 133:17-21, von Heijne (1986) *Nucleic Acids Res.* 14:4683-4690.

II. Organoid Establishment and Expansion.

[0181] Lacrimal glands and salivary glands from wildtype C57BL/6 mice were dissected and stored in Advanced DMEM/F12 (GIBCO) on ice. Multiple murine lacrimal glands or salivary glands were pooled in a petri dish and muscle, ducts and connective tissue were

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removed and discarded as much as possible. The remaining glandular epithelium was chopped into approximately 1mm pieces using a scalpel. The tissue pieces were enzymatically digested in collagenase (Sigma-Aldrich, C9407, 1 mg/mL) solution containing 10 μM ROCK inhibitor Y-27632 (Abmole, M1817) in Advanced DMEM/F12 (GIBCO) for about 15 minutes shaking at 37°C. The homogeneous cell suspension was pelleted (1200 rpm for 5 minutes) and washed twice with Advanced DMEM/F12 prior to plating. The same protocol was applied to human post-mortem lacrimal gland material. Cells were plated in 20 μL droplets of Cultrex Pathclear Reduced Growth Factor Basement Membrane Extract (BME) (3533-001, Amsbio) or Matrigel™ GFR Membrane Matrix (Corning CB40230C). After a 15-minute solidification, expansion medium containing various mammalian growth factors and cytokines, RSPO1, and a surrogate WNT (L-F12578; L-F127; L-F58; L-F4; or L-F10 at 5nM) was added. As shown in Fig. 1, cells treated with L-F12578, L-F127 or L-F58 expanded rapidly with a solid budding morphology, whereas cells treated with the control and other surrogate WNT treatments had little growth or a ductal morphology (see, e.g., Bannier-Helaouet, et al. (2021) *Cell Stem Cell* 28:1221-1232).

III. Surrogate WNT Screening in Acinar Cells.

[0182] For outgrowth activity screening of surrogate WNTs, i.e., WNT mimetics, mouse lacrimal gland or salivary gland primary cells or organoid cells (< passage 5) were digested into a near single-cell suspension using TrypLE (Thermo Fisher) for 10 minutes at 37°C. The base medium for activity assays consisted of expansion medium without RSPO1 or any surrogate WNT and supplemented with 1μM porcupine inhibitor Wnt-C59 (#5148 Tocris) and 10μM Y-27632 (#5092280001 MilliporeSigma). Experimental conditions consisted of one or a combination of 500 ng/mL recombinant RSPO1 and 5 nM surrogate WNT (L-F12578, L-F127, L-F58, L-F4, or L-F10), unless otherwise stated. All cells for all conditions were plated in 15μL Matrigel droplets in 96-well plates and submerged in 120μL of the experimental medium in round bottom 96-well plate. Cells were grown out as organoids for 7 days prior to quantification. Outgrowth efficiency was quantified using cell viability assay CellTiter-Glo® (G9683 Promega), measured on the SpectraMax Paradigm microplate reader (Molecular Devices) according to manufactures protocols, and the results are shown in Figs. 4 and 5. For RSPO dependency assay, organoids were cultures with a dose-range of surrogate WNT (0.05, 0.5, 5, 50 nM) with or without 500 ng/mL RSPO1, and the results are shown in Fig. 6.

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[0183] For WNT target gene expression screening upon surrogate WNT stimulation, mouse acinar cell organoids were grown in complete expansion days for 5 days prior to withdrawal of RSPO1, surrogate WNT and addition of 1μM porcupine inhibitor Wnt-C59 (#5148 Tocris) for 48 hours, as shown in Fig. 7. After the WNT withdrawal period, RSPO1 and/or 5 nM surrogate WNT (L-F12578, L-F127, L-F58, L-F4, or L-F10) were reintroduced for the indicated time period to assess WNT target gene induction, as shown in Fig. 8. RNA was extracted using the QIAprep miniprep kit (#27104 QIAGEN) according to manufacturer's protocol. Expression of *Axin2* was determined by qPCR using SYBR Green (#K0243 Thermo Scientific) according to manufacturer's protocol, and the results are shown in Fig. 9.

[0184] Human *AXIN2* induction in Surrogate WNT screen was performed in human lacrimal gland explant cultures, derived from fresh human lacrimal gland tissue (shown in Fig. 10), in complete expansion medium. Cells were plated in Matrigel as described above (see FIGs. 11A-11B), and exposed to different surrogate WNTs (L-F12578, L-F127, L-F58, L-F4, or L-F10) in combination with RSPO1 from the moment of plating. After 24-hour exposure, the RNA was extracted using the QIAGEN miniprep kit according to manufacturer's protocol. Expression of *AXIN2* was determined by qPCR using SYBR Green (Thermo Fisher) according to manufacturer's protocol (primers sequences provided below), and the results are shown in Fig. 12.

IV. Immunofluorescence Staining and In Situ Hybridization.

[0185] Organoids were harvested in cell recovery solution (354253, Corning) and fixed in 4% formaldehyde (R37814 Sigma-Aldrich) for at least 2 hours at room temperature and permeabilized using 0.2% Triton X-100 in PBS (#ICN19485450 Fisher Scientific). Whole-mount staining was performed overnight in 2% donkey serum using rabbit anti-MIST1 (#14896 CST) and DAPI (#EN62248 Fisher Scientific), secondary antibody was Alexa Fluor 568 donkey anti-rabbit IgG (Life Technologies, A10042). Organoids were imaged on a Leica THUNDER imaging system (FIG. 2). Immunofluorescence staining of murine lacrimal glands was done on sections from paraffin embedded tissue. In short, sections were deparaffinized, heat antigen retrieval, permeabilized, blocked, and stained for Ki67 (# ab15580 Abcam) and DAPI (#EN62248 Fisher Scientific). Secondary staining was performed using the secondary antibody, Alexa Fluor 568 donkey anti-rabbit IgG (Life Technologies, A10042), and imaging was performed on a Leica DMi8 system (Fig. 15). In situ hybridizations were performed according to manufactures protocol (ACDBio) with

human or mouse specific probes for indicated WNT receptors and imaged on a Leica DMi8 system. The results are shown in Figs. 13-14.

V. Animals and Treatment

[0186] Female C57BL/6 mice (8–10-week-old) were intraperitoneally dosed on day 0, 3, 7 and 10 at 10 mpk with various Wnt mimetics. At day 14, proliferating cells were detected in L-F12578, L-F127 and L-F58 treatment groups by Ki67 signal (FIG. 15). A similar timing of dosing with WNT mimetics in combination with RSPO resulted in a lacrimal gland weight increase in the L-F12578 and L-F127 treatment groups after two weeks compared to control and RSPO alone (FIG. 16).

[0187] Female C57BL/6 mice (8–10-week-old) were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed in accordance with IACUC committee's regulations. Mice were anesthetized and the left extraorbital lacrimal glands were left untreated while the right lacrimal glands were injected with IL-1α or a cocktail of IL-1α and SWAP™, i.e., WNT mimetic, as shown in FIG. 17.

[0188] All animal experiments were performed according to national ethical guidelines in addition to the guidance and approval by the Institutional Animal Care and Use Committee (IACUC) of Surrozen, Inc. Twelve-week-old MRL-*lpr* (stock 000485) female mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were housed 4 per cage. Proteins treatments at 3mg per kg and 10 mg per kg were intraperitoneally dosed on day 0, 3, 7 and 10 in C57Bl/6J mice and MRL-*lpr* along with MRL/MpJ mice respectively. RSPO2-nFcFc treatment at 0.1, 0.3, 1 and 3mg per kg were intraperitoneally dose on day 0, 3, 7 and 10 in C57Bl/6J mice. Bone mineral density (BMD) and fat content of animals were measured via *in vivo* DEXA method using a Faxitron UltraFocus (Faxitron Bioptics, Tucson, Arizona) on day 0, 7 and 13. Animals were anesthetized during imaging through isoflurane and sample ROI included the entire murine skeleton except material above the cervical spine due to increased radiographical intensity of the skull. BMD and fat content were calculated using the accompanying Vision DXA software. Animals were terminated on day 14, and salivary glands were collected for histology.

VI. Intra-Lacrimal Gland Injection

[0189] Intra-lacrimal gland injection was performed as previously described with minor alteration in Zoukhri et al. A Single Injection of Interleukin-1 Induces a Reversible Aqueous-tear Deficiency, Lacrimal Gland Inflammation, and Acinar and Ductal Cell Proliferation, 84

EXP. EYE RES. 894-904 (2007). Briefly, a small incision anterior to the ear of isoflurane-anesthetized animal was made to expose the extraorbital lacrimal gland. A volume of 1 μL was injected 3 times (total volume of 3 μL) to the exposed lacrimal gland.

[0190] The following procedure was established for lacrimal gland duct ligation, adapted from Liu et al. 2017. Animals are anesthetized with isoflurane. After the mouse is no longer responsive to pinches on two extremities in two different body quadrants, it is moved to the stage of a dissecting scope and fashioned with a nosecone to continue anesthesia by isoflurane. A small incision anterior to the ear is made to expose the extraorbital lacrimal gland which is lifted to expose the main excretory duct. A size 4-0 silk thread is then used to ligate the duct. After duct ligation, the skin incision is closed, sutured, and antibiotic ointment is then applied to the skin. The contralateral lacrimal gland is not operated on and served as a control. After three days, the skin suture is reopened to expose the lacrimal gland. The duct ligation is then released by cutting the silk suture with micro scissors. In the same surgery, treatment groups are injected with 2 mL of test article into the lacrimal gland ipsilateral to the ligation. After injection, the skin incision is closed, sutured, and antibiotic ointment is then applied. The animals are then sacrificed, and ipsilateral and contralateral lacrimal glands collected 24h, 3 days or 14 days after releasing the ligation.

VII. Induction of Dry Eye and SWAP™ Treatment

[0191] For induction of dry eye, 3 μL of recombinant human IL-1α (Peprotech) at 2 μg/μL was injected to the lacrimal gland. For local SWAP™ treatment, SWAP™ (3.5 μg/μL) was mixed with IL-1α (2 μg/μL) in a total volume of 3 μL then injected to the lacrimal gland. For systemic SWAP™ treatment, SWAP™ was intraperitoneally injected at 10 mg/kg twice a week for 2 weeks, as shown in Figs. 19-20.

VIII. Measurement of Aqueous Tear Secretion

[0192] Aqueous tear secretion was measured using phenol-impregnated cotton threads (Zone-Quick, Menicon) on restrained, unanesthetized mice. The threads were held with forceps and applied to the lateral canthus of both eyes for 30 seconds. Wetting of the thread which turned from yellow to red in contact with tears was measured in millimeters, and the results are shown in Fig. 19.

IX. WNT Mimetic Molecules Induce Salivary Gland Hypertrophy

[0193] The effect of systemic WNT mimetic administration on salivary gland weight and histology *in vivo*, was examined. Significant increases in salivary gland weight in the groups of L-F12578, L-F127, L-F4, and L-F10 dosed at 3 mpk compared with Vehicle group on day 14, by 101% ($P<0.001$), 114% ($P<0.001$), 29% ($p<0.01$) and 22%($P<0.05$), respectively, were observed, with the effects of L-F12578 and L-F127 being the most pronounced (Fig. 22). None of the WNT mimetic molecules, targeting different FZD receptors, had any observable effect on the histology of the submandibular gland *in vivo* by H&E stain (Fig. 23A). Staining for proliferative cells by Ki67 showed little difference between treatment groups, indicating a potential earlier peak of cell division driving the differences in organ weight (Fig. 23B). Animals dosed with the same molecules but at 10 mpk (for 14 days, bi-weekly systemic treatment) did have a clear histopathology phenotype. L-F12578 and L-F127 treated animals displayed a difference in staining in the seromucinous glandular acini with an increase in the amount of cytoplasmic basophilia determined by an independent pathologist (Fig. 24A). Automated quantification of the mucinous and serous portion of the salivary gland by Image J shows a significant reduction in the mucinous acini area in L-F12578 and L-127 treated animals (Fig. 24B). Also, systemic RSPO2 administration elicited a hypertrophic response in the salivary gland. In a two-week treatment with a range of RSPO2-nFc doses the 1 mpk and 3 mpk dose have a significant increase in salivary gland weight at day 14 (Fig. 25).

[0194] To understand the effect of WNT activation on salivary gland cells, murine salivary gland organoids as an experimental platform (see, e.g., Maimets et al. (2016) *Stem Cell* 6:150-162) were utilized. Treatment with L-F12578 (5nM) in combination with recombinant RSPO1 increased proliferation compared to RSPO1 alone, resulting in the fast expansion of larger organoids (Fig. 26A). FZD specificity of this pro-proliferation phenotype was examined by screening the WNT mimetics in an outgrowth assay and compared it to anti-GFP antibody. In a dose-dependent manner, L-F12758 and L-F127 significantly increased the outgrowth efficiency measured as cell viability at day 10, and a modest effect was observed for L-F58 at high concentrations (Fig. 26B). *Fzd7*, *Fzd1* and *Fzd2* were shown to be the highest expressed Frizzled's in murine salivary gland organoids consistent with the *in vitro* and *in vivo* effects on salivary gland (Fig. 27).

[0195] Sjögren's syndrome is a systemic autoimmune disease that particularly affects exocrine glands such as the salivary glands. Chronic inflammation in the glands with immune cell infiltration results in acinar cell atrophy leading to xerostomia (see, e.g., Jensen and

Vissink (2014) *Oral Maxillofac. Surg. Clin. North Am.* 26:35-53). The MRL-*lpr* mouse strain, also known as lupus mice, exhibit systemic autoimmunity with similar salivary gland abnormalities in Sjögren's syndrome in humans such as reduced salivary production and lymphocyte infiltration (see, e.g., Ma et al. (2014) *Diag. Pathol.* 9:5). To test whether the L-F12578 WNT mimetic can increase and restore salivary gland weight in an ongoing damage environment, aged lupus and MRL/*MpJ* control mice were dosed for two weeks. Treatment with L-F12578 significantly increased salivary gland weight in both genetic strains without any observable adverse effect on histology (Figs. 28A and 28B). Both the lupus mice and the control strain exhibited wet fur with mild color change upon treatment with L-F12578.

X. Reagents and Materials

[0196] WNT mimetics were constructed as described in WO 2020/010308 A1 incorporated herein in its entirety herein. All recombinant proteins were produced in Expi293F™ cells (Thermo Fisher Scientific) by transient transfection unless otherwise specified. All IgG-based and Fc-containing constructs were first purified with Protein-A resin and eluted with 0.1 M glycine pH 3.5. All proteins were then polished by a size exclusion column in HBS buffer (10 mM HEPES pH 7.2, 150 mM NaCl). Proteins were supplemented with glycerol to 10% for long term storage at -80°C.

[0197] Reagents used in lacrimal gland regeneration experiments include (but are not limited to):

Reagent List	
Component	*Catalog number*
Advanced DMEM/F-12	Thermo Scientific 12634-010
HEPES	Thermo Scientific 15630080
GlutaMAX	Thermo Scientific 35050061
Penicillin-Streptomycin	Thermo Scientific 15140122
B27	Thermo Scientific 17504044
N2	Thermo Scientific 17502048
Human Growth Factors	Peprotech
Recombinant Human R-Spondin 1	R&D 4645RS
Surrogate WNT	In-house production
Matrigel™ GFR Membrane Matrix	Corning CB40230C
Y-27632	MilliporeSigma 5092280001

[0198] Primers used in the experiments include:

Primer name	*Primer sequence*	*SEQ ID No.*
Mouse forward *Axin2*	CAGCCCAAGAACCGGGAAAT	17
Mouse reverse *Axin2*	GAGCCTCCTCTCTTTTACAGCA	18
Mouse forward *Actb*	ACCTTCTACAATGAGCTGCGT	19
Mouse reverse *Actb*	AGGTCTCAAACATGATCTGGGT	20
Mouse forward Rnf43	CGGAGCCGTACTGGGTTAAG	21
Mouse reverse Rnf43	GGCACGGGGATGAAACAAAT	22
Mouse forward Mist1	CCAAGATCGAGACCCTCACG	23
Mouse reverse Mist1	GCGGCTGCTGGACATAGTAA	24
Mouse forward Krt7	GCGGAGATGAACCGCTCTAT	25
Mouse reverse Krt7	TCTAACTTGGCACGCTGGTT	26
Human forward *AXIN2*	TCATTTCCCGAGAACCCACC	27
Human reverse *AXIN2*	AGCTTCAGCTTTTCCAGCCT	28
Human forward *ACTB*	CTGGAACGGTGAAGGTGACA	29
Human reverse *ACTB*	AAGGGACTTCCTGTAACAATGCA	30
Mouse forward Fzd1	CATCGAGGCCAACTCACAGT	31
Mouse reverse Fzd1	TGAGCCCCACAAAACACACT	32
Mouse forward Fzd2	AGCGATTTCGCTACCCAGAG	33
Mouse reverse Fzd2	AGAACGAAGCCCGCAATGTA	34
Mouse forward Fzd3	GCCCTTTGTGAGACCAGGTT	35
Mouse reverse Fzd3	ATATGCCTGCAGGTCTCAGC	36
Mouse forward Fzd4	CCTGAGCGCCCCATCATATT	37
Mouse reverse Fzd4	CTTTCCCGGCCTACAGTCAG	38
Mouse forward Fzd5	CCGCCACAGGTACCTAGCTT	39
Mouse reverse Fzd5	AAGGACAGAACTCTCGGAGGA	40
Mouse forward Fzd6	CCCGAGGTGAGGCGTCC	41
Mouse reverse Fzd6	TGAATTCTGGGGCAACTGCT	42
Mouse forward Fzd7	ATGCTCTATGTACGCACCCG	43
Mouse reverse Fzd7	CGAACTTGTTCATGAGCGCC	44
Mouse forward Fzd8	TGTTGGAAGTGACCTCGCTC	45
Mouse reverse Fzd8	AACCGATGCCTTTGCACAAC	46

Mouse forward Fzd9	TAAGGACTTCGCGCTGGTTT	47
Mouse reverse Fzd9	AAGATAATCGGGCGCTCTGG	48
Mouse forward Fzd10	CCTTCCTCATCGACCCATCG	49
Mouse reverse Fzd10	TAGCCCACCGAATAAACGCA	50
Mouse forward Lrp5	CCATTGTGTTGCACCCTGTG	51
Mouse reverse Lrp5	CAGGACATGCCGATCTCTCC	52
Mouse forward Lrp6	TGCAAACAGACGGGACTTGA	53
Mouse reverse Lrp6	CCAAACACAAAGTCCACCGC	54

[0199] WNT mimetic constructs used in the experiments comprise the following amino acid sequences (Bold = LRP binding variable domain; underline = linker; italics = FZD binding variable domain; Plain (non-bold, non-underline, and non-italics = Fc domain).

L-F12578 (Fv-IgG)	Heavy Chain SEQ ID: 1 **EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVA** **EISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALR** **ARPPIRLHPRGSVMDYWGQGTLVTVSS**GGGGSGGGGSGGGGS*EVQLVESG* *GGLVQPGGSLRLSCAASGFTFSHYTLSWVRQAPGKGLEWVSVISGDGSYTYYADSV* *KGRFTISSDNSKNTLYLQMNSLRAEDTAVYYCARNFIKYVFANWGQGTLVTVSS*AST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALG APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK Light Chain SEQ ID: 2 **DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYS** **ASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGT** **KVEIK**GGGGSGGGGSGGGGS*DIELTQPPSVSVAPGQTARISCSGDNIGSFYVHWY* *QQKPGQAPVLVIYDKSNRPSGIPERFSGSNSGNTATLTISGTQAEDEADYYCQSYAN* *TLSLVFGGGTKLTVL*GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTV AWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHE GSTVEKTVAPTECS
L-F127 (Fv-Ig)	Heavy Chain SEQ ID: 3 **EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVA** **EISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALR** **ARPPIRLHPRGSVMDYWGQGTLVTVSS**GGGGSGGGGSGGGGS*EVQLVESG* *GGLVQPGGSLRLSCAASGFNISSSYIHWVRQAPGKGLEWVAYIYSSYGSTYYADSVK* *GRFTISADTSKNTAYLQMNSLRAEDTAVYYCARASWYALDYWGQGTLVTVSS*ASTK GPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP PCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGA PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESN GQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK Light Chain SEQ ID: 4 **DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYS** **ASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGT** **KVEIK**GGGGSGGGGSGGGGS*DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAW* *YQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQYWY* *GVAPITFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC

L-F58 (Fv-IgG)	Heavy Chain SEQ ID: 5 **EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVA** **EISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALR** **ARPPIRLHPRGSVMDYWGQGTLVTVSS**GGGGSGGGGSGGGGS*EVQLVESG* *GGLVQPGGSLRLSCAASGFNISYSYIHWVRQAPGKGLEWVASIYSSSGSTSYADSVK* *GRFTISADTSKNTAYLQMNSLRAEDTAVYYCARGAIDYWGQGTLVTVSS*ASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIE KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT QKSLSLSPGK Light Chain SEQ ID: 6 **DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYS** **ASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGT** **KVEIK**GGGGSGGGGSGGGGS*DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAW* *YQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQWYS* *SGHVLITFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEV THQGLSSPVTKSFNRGEC

L-F4 (Fv-IgG)	Heavy Chain SEQ ID: 7 **EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVA** **EISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALR** **ARPPIRLHPRGSVMDYWGQGTLVTVSS**GGGGSGGGGSGGGGS*EVQLVESG* *GGLVQPGGSLRLSCAASGFNISYYYIHWVRQAPGKGLEWVASIYPSSGYTYYADSVK* *GRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSSFYWAMDYWGQGTLVTVSS*AST KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALG APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGK Light Chain SEQ ID: 8 **DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYS** **ASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGT** **KVEIK**GGGGSGGGGSGGGGS*DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAW* *YQQKPGKAPKLLIYSASSLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQSYA* *AYLFTFGQGTKVEIK*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC

L-F10 (Fv-IgG)	Heavy Chain SEQ ID: 9 **EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVA** **EISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALR** **ARPPIRLHPRGSVMDYWGQGTLVTVSS**GGGGSGGGGSGGGGS*EVQLVESG* *GGLVQPGGSLRLSCAASGFTFSSFNMFWVRQAPGKGLEWVAGIDDDGSYPNYGS* *AVKGRATISRDNSKNTLYLQMNSLRAEDTAVYYCAKSGYGGSWGGYIADDIDAWG* *QGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK Light Chain SEQ ID: 10 **DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYS** **ASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGT** **KVEIK**GGGGSGGGGSGGGGS*ELTQPPSVSVSPGQTARITCSGDGSYAGSYYYGW* *YQQKPGQAPVTLIYYNNKRPSGIPERFSGSLSGSTNTLTISGVQAEDEADYYCGSAD* *NSGAAFGGGTKLTVL*RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT HQGLSSPVTKSFNRGEC
1SH1-03	Light Chain SEQ ID: 11 **DVQLVESGGGLVQPGGSLRLSCTSSANINSIETLGWYRQAPGKQRELIAN** **MRGGGYMKYAGSLKGRFTMSTESAKNTMYLQMNSLKPEDTAVYYCYV** **KLRDDDYVYRGQGTQVTVSS**GGSGS*DVVMTQSPLSLPVTPGEPASISCRSSQSL* *LHSNGYNYLDWYLQKPGQSPQLLIYLGSKRASGVPDRFSGSGSGTDFTLKISRVEA* *EDVGVYYCMQALQIPPTFGQGTKVEIKR*TVAAPSVFIFPPSDEQLKSGTASVVCL LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYE KHKVYACEVTHQGLSSPVTKSFNRGEC
1SH1-26	Light Chain SEQ ID: 12 **DVQLVESGGGLVQAGGSLRLACAGSGRIFAIYDIAWYRHPPGNQRELVA** **MIRPVVTEIDYADSVKGRFTISRNNAMKTVYLQMNNLKPEDTAVYYCNA** **KRPWGSRDEYWGQGTQVTVSS**GGSGS*DVVMTQSPLSLPVTPGEPASISCRSSQ* *SLLHSNGYNYLDWYLQKPGQSPQLLIYLGSKRASGVPDRFSGSGSGTDFTLKISRVE* *AEDVGVYYCMQALQIPPTFGQGTKVEIKR*TVAAPSVFIFPPSDEQLKSGTASVVC LLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSFNRGEC
1SH1-36	Light Chain SEQ ID: 13 **QVKLEESGGGLVQAGGSLRLSCAASGRIFSIYDMGWFRQAPGKEREFVS** **GIRWSGGTSYADSVKGRFTISKDNAKNTIYLQMNNLKAEDTAVYYCGSR** **GYWGQGTLVTVSS**GGSGS*DVVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYN* *YLDWYLQKPGQSPQLLIYLGSKRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYY* *CMQALQIPPTFGQGTKVEIKR*TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR

	EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYA CEVTHQGLSSPVTKSFNRGEC
1SH1-03 1SH1-26 1SH1-36	Heavy Chain SEQ ID: 14 *EVQLVESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSG STYYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCARHAGFYGLADYFDYWG QGTLVTVSS*ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV EPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPGK
L-F49	Light Chain SEQ ID: 15 **DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGT KVEIKR***GGGGSGGGGSGGGGS*DIQMTQSPSSPSASVGDRVTITCRASQSISSYL NWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYY CQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY ACEVTHQGLSSPVTKSFNRGEC Heavy Chain SEQ ID: 16 **EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYYISWVRQAPGKGLEWVA EISPYSGSTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCALR ARPPIRLHPRGSVMDYWGQGTLVTVSS***GGGGSGGGGSGGGGS***EVQLVQS GAEVKKPGASVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGWINAGN GNTTYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARHYYGSG SYPDWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS NTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[0200] It will be readily understood to those skilled in the art that the above constructs may be altered and expressed as various homologs and isoforms, may be edited at non-binding-domain sequences, and may be expressed using various synonymous nucleotide sequences using various suitable expression vector systems.

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Xie, Y., Zamponi, R., Charlat, O., Ramones, M., Swalley, S., Jiang, X., Rivera, D., Tschantz, W., Lu, B., Quinn, L., et al. (2013). *Interaction with both ZNRF3 and LGR4 is required for the signalling activity of R-spondin.* EMBO Rep *14*, 1120-1126. 10.1038/embor.2013.167.

IN THE CLAIMS:

1. A method of regenerating lacrimal gland cells in a subject, comprising administering a WNT signaling modulator to the subject.

2. The method of claim 1 wherein the lacrimal gland cells are acinar cells, progenitor cells, ductal cells, myoepithelial cells, or immune cells.

3. The method of claim 1, wherein the WNT signaling modulator is an engineered WNT signaling modulator.

4. The method of claim 3, wherein the engineered WNT signaling modulator is a WNT agonist.

5. The method of claim 1, wherein the WNT signaling modulator is an engineered WNT superagonist comprising an E3 Ligase binding domain selected from the group consisting of: a mutant R-spondin (RSPO) protein; and an antibody specific for E3 ligase or functional fragment thereof.

6. The method of claim 1, wherein the WNT signaling modulator comprises at least one engineered bi-specific IgG antibody or antigen binding fragment thereof that directly activates a canonical WNT signaling pathway.

7. The method of claim 6, wherein the bi-specific IgG antibody or antigen binding fragment thereof comprises a binding composition specific for at least one FZD receptor and a binding composition specific for at least one LRP receptor.

8. The method of claim 1, wherein the WNT signaling modulator is selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic;

a) the FZD binding composition binds to at least one of Fzd1, Fzd2, Fzd5, Fzd7, Fzd8; and

b) the LRP binding composition binds to at least one of Lrp6, and/or Lrp5.

9. The method of claim 1, wherein the WNT signaling modulator binds to the group consisting of: Fzd5 and Fzd8, and Lrp6 and/or Lrp5.

10. The method of claim 1, wherein the WNT signaling modulator comprises one or more polypeptides selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID: NO 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO:15, SEQ ID NO:16, and any isoform or homolog thereof.

11. The method of claim 10 wherein the WNT signaling modulator comprises:

a) two polypeptides of SEQ ID NO:1 and two polypeptides of SEQ ID NO:2, or variants thereof having at least 90% identity thereto;

b) two polypeptides of SEQ ID NO:3 and two polypeptides of SEQ ID NO:4, or variants thereof having at least 90% identity thereto;

c) two polypeptides of SEQ ID NO:5 and two polypeptides of SEQ ID NO:6, or variants thereof having at least 90%identity thereto;

d) two polypeptides of SEQ ID NO:7 and two polypeptides of SEQ ID NO:8, or variants thereof having at least 90% identity thereto; or

e) two polypeptides of SEQ ID NO:9 and two polypeptides of SEQ ID NO:10, or variants thereof having at least 90% identity thereto;

f) two polypeptides of SEQ ID NO:11 and two polypeptides of SEQ ID NO:14, or variants thereof having at least 90% identity thereof;

g) two polypeptides of SEQ ID NO:12 and two polypeptides of SEQ ID NO:14, or variants thereof having at least 90% identity thereof;

h) two polypeptides of SEQ ID NO:13 and two polypeptides of SEQ ID NO:14, or variants thereof having at least 90% identity thereof; or

i) two polypeptides of SEQ ID NO:15 and two polypeptides of SEQ ID NO:16, or variants thereof having at least 90% identity thereof.

12. The method of any of claims 1-11 wherein the method further comprises co-administration of at least one molecule from the group consisting of: RSPO2, RSPO2 fragment, and engineered RSPO2 mimetic.

13. The method of any of claims 1–12 wherein the subject is a mammal.

14. The method of any of claims 1–13 wherein the subject is a human.

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15. The method of any of claims 1-14 wherein the subject is in need of lacrimal cell regeneration because of a lacrimal gland disorder.

16. The method of claim 15, wherein the lacrimal gland disorder is a dry eye disease.

17. The method of claim 16, wherein the dry eye disease is caused by Sjögren's syndrome, chronic graft versus host disease (cGHVD), rheumatoid arthritis (RA), Stephen's Johnson syndrome, ocular rosacea, chemotherapy, radiation oncology treatments, diabetes, lupus, or a Meibomian Gland Disorder (MGD).

18. The method of claim 17, wherein the dry eye disease is Sjögren's syndrome.

19. A method of treating a lacrimal gland disorder in a subject, comprising administering a WNT signaling modulator to the subject.

20. The method of claim 19, wherein the WNT signaling modulator is an engineered WNT signaling modulator.

21. The method of claim 19, wherein the WNT signaling modulator is an engineered WNT agonist.

22. The method of claim 19, wherein the WNT signaling modulator comprises at least one engineered bi-specific antibody or antigen binding fragment thereof that directly activates a canonical WNT signaling pathway.

23. The method of claim 19, wherein the engineered WNT agonist is selected from: (*i*) WNT3a; (*ii*) a WNT mimetic; or (*iii*) an R-spondin mimetic.

24. The method of claim 19, wherein the WNT signaling modulator binds to:
a) any one or more of the group consisting of: Fzd1, Fzd2, Fzd5, Fzd7, Fzd8; and
b) Lrp6, and/or Lrp5.

25. The method of claim 19, wherein the WNT signaling modulator binds to:
a) any one or more of the group consisting of: Fzd1, Fzd2, and Fzd7; and
b) Lrp6, and/or Lrp5.

26. The method of claim 20, wherein the WNT signaling modulator binds to:

a) any one or more of the group consisting of: Fzd5 and Fzd8; and

b) Lrp6 and/or, Lrp5.

27. The method of claim 19, wherein the WNT signaling modulator comprises a sequence selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID: NO 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and any isoform or homolog thereof of any of the foregoing, optionally wherein the WNT signaling modulator comprises:

a) two polypeptides of SEQ ID NO:1 (or variants of one or both having at least 90% identity thereto) and two polypeptides of SEQ ID NO:2 (or variants of one or both having at least 90% identity thereto);

b) two polypeptides of SEQ ID NO:3 (or variants of one or both having at least 90% identity thereto) and two polypeptides of SEQ ID NO:4 (or variants of one or both having at least 90% identity thereto);

c) two polypeptides of SEQ ID NO:5 (or variants of one or both having at least 90% identity thereto) and two polypeptides of SEQ ID NO:6 (or variants of one or both having at least 90% at least identity thereto);

d) two polypeptides of SEQ ID NO:7 (or variants of one or both having at least 90% identity thereto) and two polypeptides of SEQ ID NO:8 (or variants of one or both having at least 90% identity thereto); or

e) two polypeptides of SEQ ID NO:9 (or variants of one or both having at least 90% identity thereto) and two polypeptides of SEQ ID NO:10 (or variants of one or both having at least 90% identity thereto);

f) two polypeptides of SEQ ID NO:11 (or variants of one or both having at least 90%, at least 95%, or at least 98% identity thereto) and two polypeptides of SEQ ID NO:14 (or variants of one or both having at least 90% identity thereto);

g) two polypeptides of SEQ ID NO:12 (or variants of one or both having at least 90% identity thereto) and two polypeptides of SEQ ID NO:14 (or variants of one or both having at least 90%, identity thereto);

h) two polypeptides of SEQ ID NO:13 (or variants of one or both having at least 90%, identity thereto) and two polypeptides of SEQ ID NO:14 (or variants of one or both having at least 90%, identity thereto); or

i) two polypeptides of SEQ ID NO:15 (or variants of one or both having at least 90%, identity thereto) and two polypeptides of SEQ ID NO:16 (or variants of one or both having at least 90%, identity thereto).

28. The method of any of claims 19-27 wherein the method further comprises administering at least one of the group consisting of: RSPO2, an RSPO2 fragment, and an engineered RSPO2 mimetic.

29. The method of any of claims 19-28 wherein the WNT signaling modulator is administered in a therapeutically effective amount.

30. The method of any of claims 19-29 wherein the subject is a live mammal.

31. The method of any of claims 19-30 wherein the subject is a human patient.

32. The method of any of claims 19-31 wherein the lacrimal gland disorder is dry eye disease.

33. The method of claim 32, wherein the dry eye disease is caused by Sjögren's syndrome, chronic graft versus host disease (cGHVD), rheumatoid arthritis (RA), Stephen's Johnson syndrome, ocular rosacea, chemotherapy, radiation oncology treatments, diabetes, lupus, or a Meibomian Gland Disorder (MGD).

34. The method of claim 33, wherein the dry eye disease is caused by Sjögren's syndrome.

35. A composition for the treatment of a dry-eye disorder in a subject, the composition comprising a WNT signaling modulator.

36. The composition of claim 35, wherein the WNT signaling modulator comprises at least one engineered bi-specific antibody or antigen binding fragment thereof that directly activates a canonical WNT signaling pathway.

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37. The composition of claim 35, wherein the at least one engineered bi-specific full-length IgG antibody or antigen binding fragment thereof is specific for or preferentially binds any one of the following combination of Fzds:

a) Fzd1, Fzd2, Fzd5, Fzd7, Fzd8;

b) Fzd 1, Fzd 2, and Fzd 7; or

c) Fzd 5 and Fzd 8,

wherein the engineered bi-specific antibody or antigen binding fragment thereof also binds Lrp 5 and/or Lrp6.

38. The composition of claim 35, further comprising an anti-inflammatory agent.

39. The composition of claims 35-38, wherein the composition comprises therapeutically effective amounts of its components.

40. The composition of claims 35-38, wherein the subject is a live mammal.

41. The composition of claims 35-38, wherein the subject is human patient.

42. The composition of claims 35-38, wherein the aqueous-deficient dry-eye disorder is caused by a Sjögren's syndrome, disorder, chronic graft versus host disease (cGHVD), rheumatoid arthritis (RA), Stephen's Johnson syndrome, ocular rosacea, chemotherapy, radiation oncology treatments, diabetes, lupus, or a Meibomian Gland Disorder (MGD.

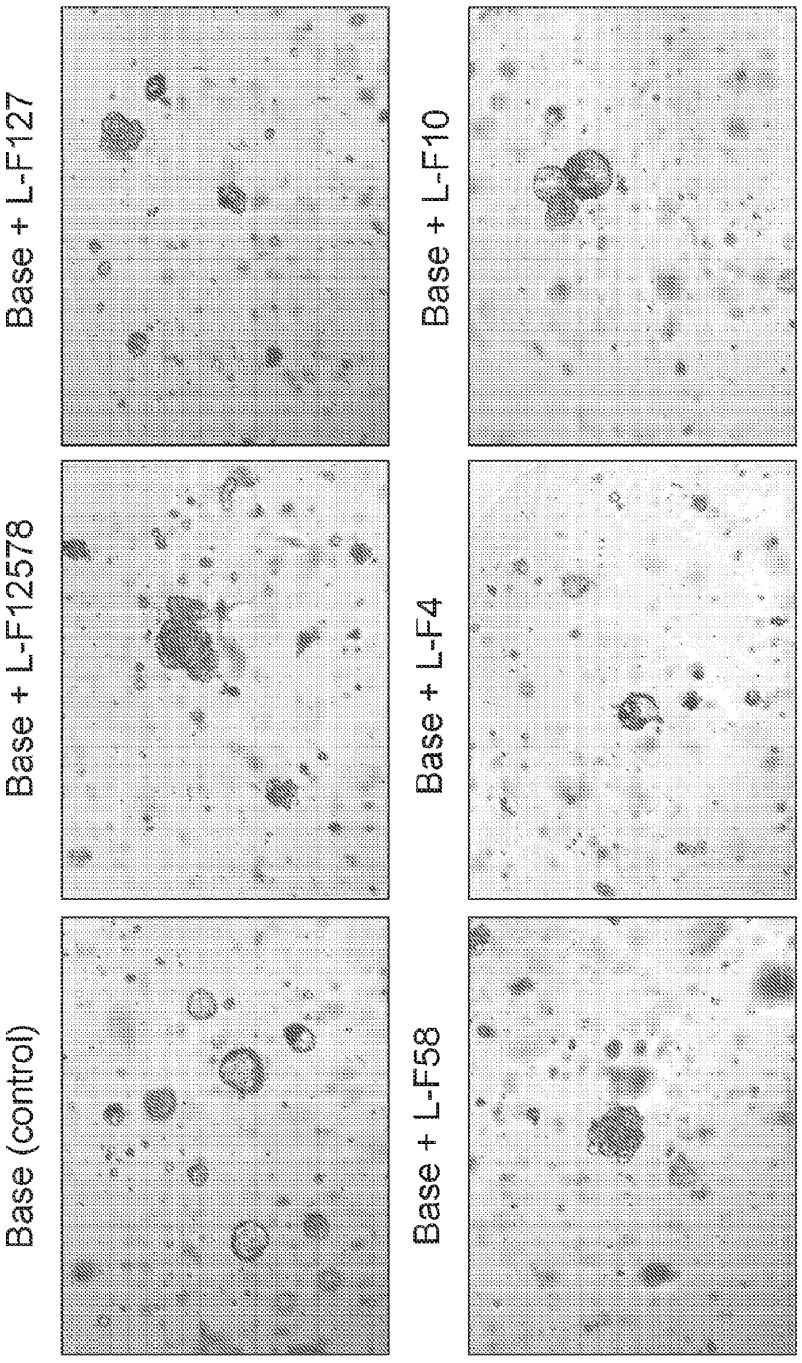

Base (control)
Base + L-F58
Base + L-F12578
Base + L-F4
Base + L-F127
Base + L-F10

FIG. 1

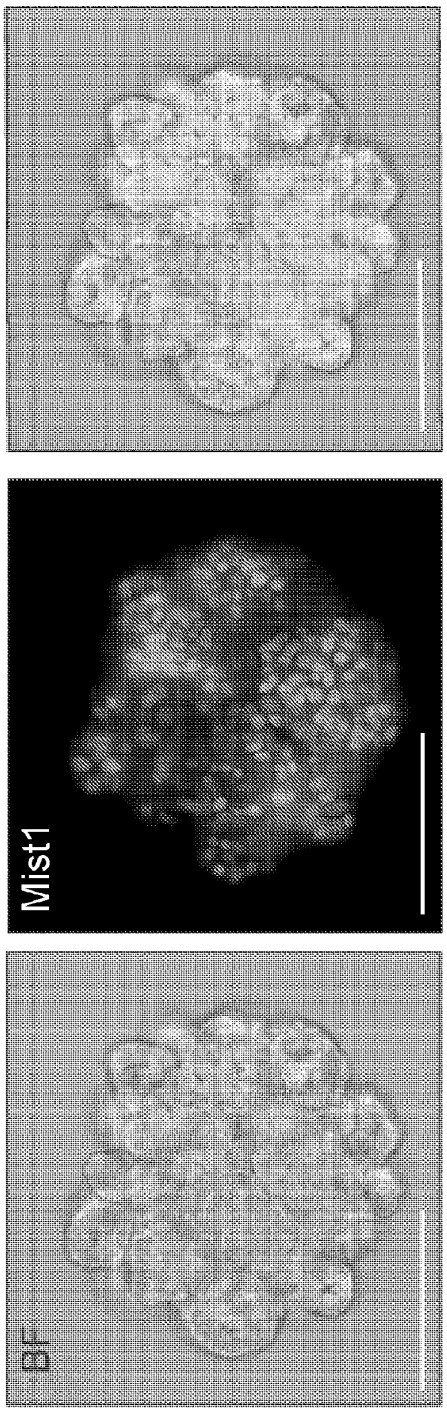


FIG. 2

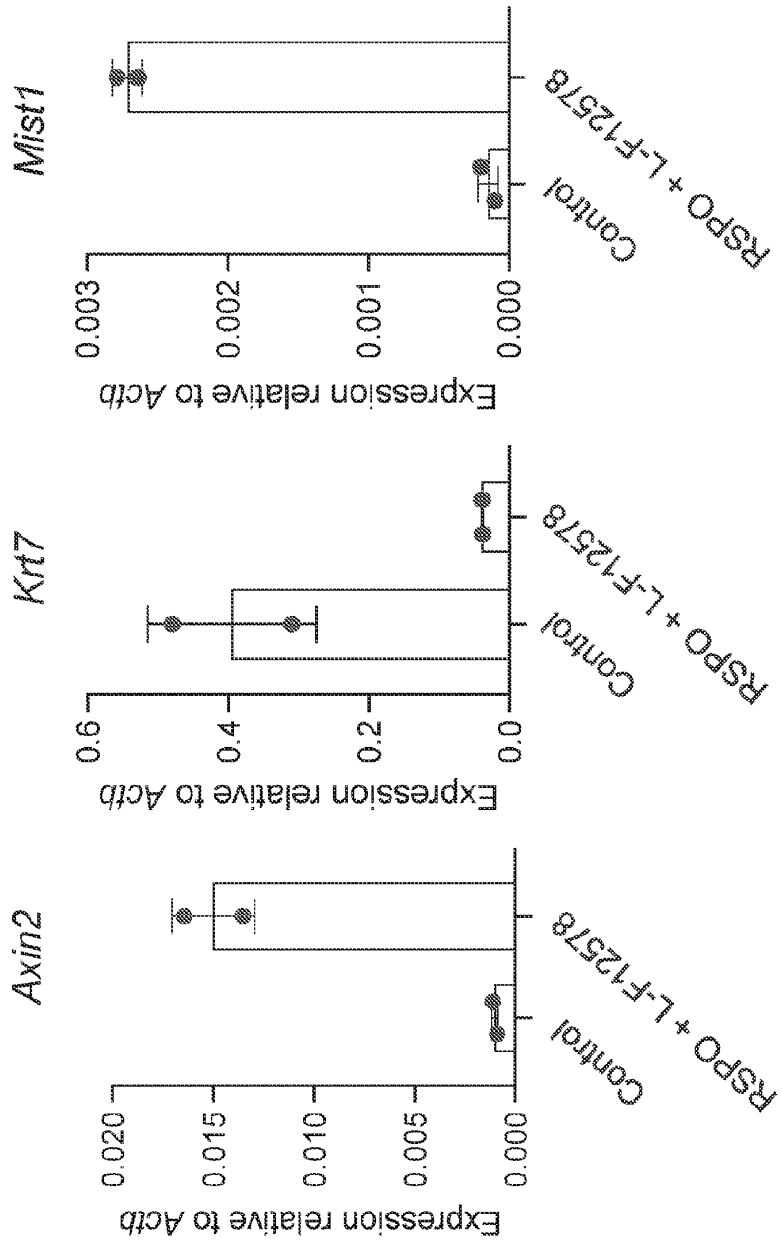


FIG. 3A

FIG. 3B

FIG. 3C

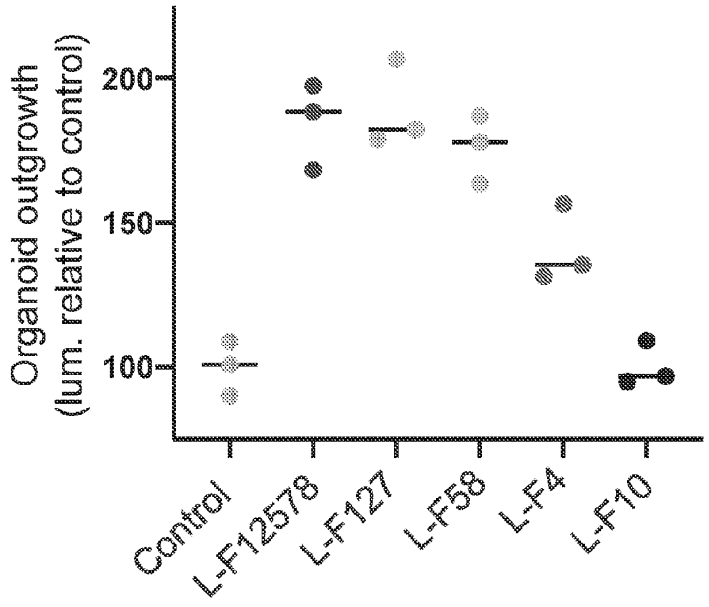

Organoid outgrowth
(lum. relative to control)
200
150
100
Control
L-F12578
L-F127
L-F58
L-F4
L-F10

FIG. 4

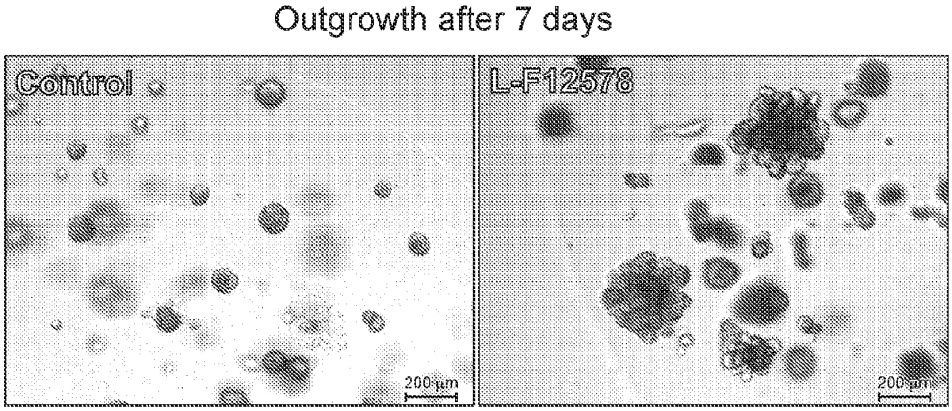

Outgrowth after 7 days
Control
L-F12578
200µm
200µm

FIG. 5

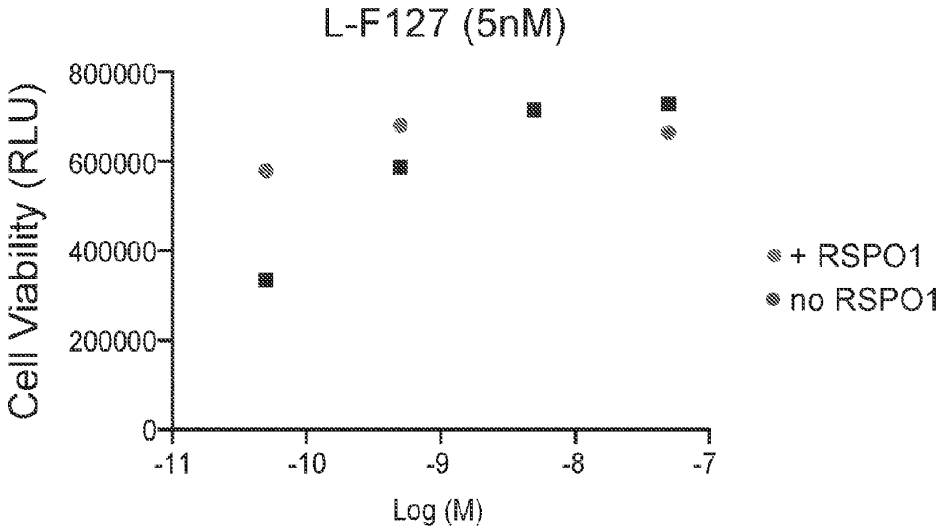

L-F127 (5nM)
Cell Viability (RLU)
800000
600000
400000
200000
0
-11
-10
-9
-8
-7
Log (M)
+ RSPO1
no RSPO1

FIG. 6

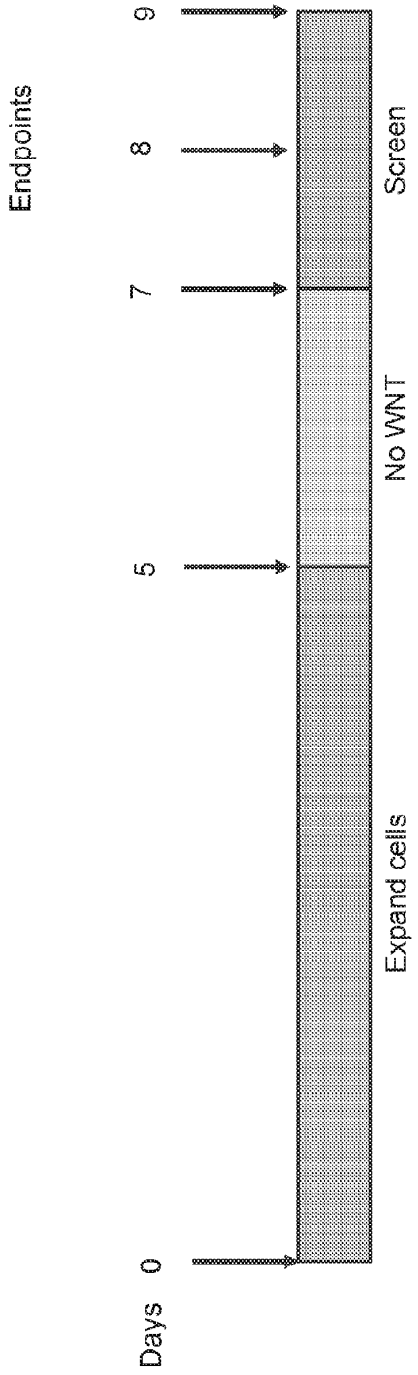


FIG. 7

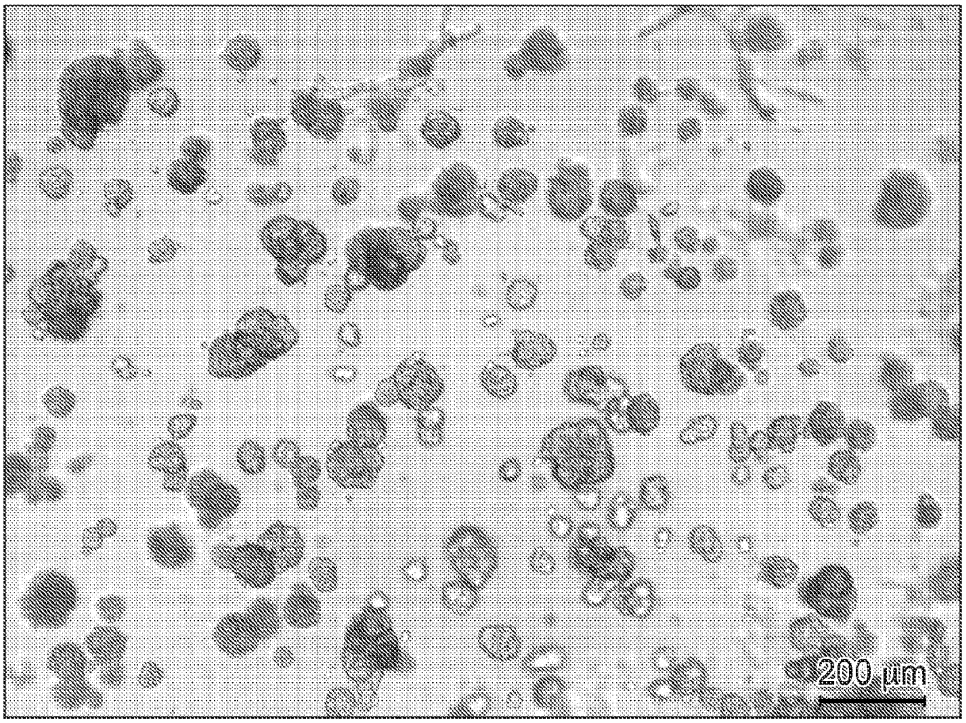


FIG. 8

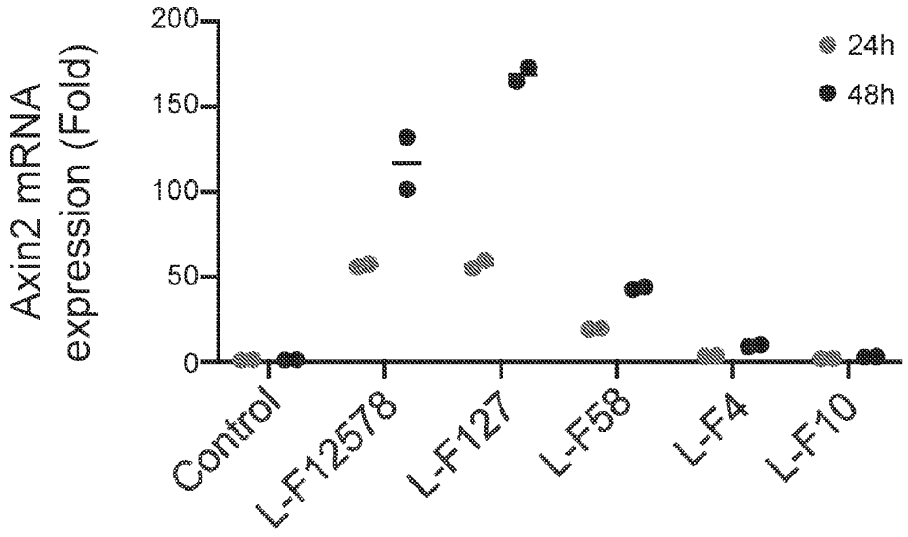

Axin2 mRNA expression (Fold)
200
150
100
50
0
24h
48h
Control
L-F12578
L-F127
L-F58
L-F4
L-F10

FIG. 9

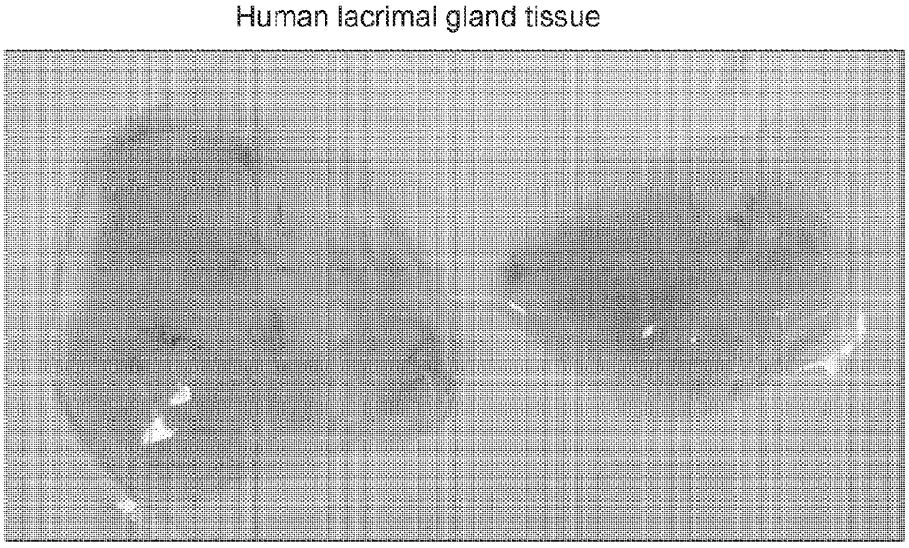

Human lacrimal gland tissue

FIG. 10

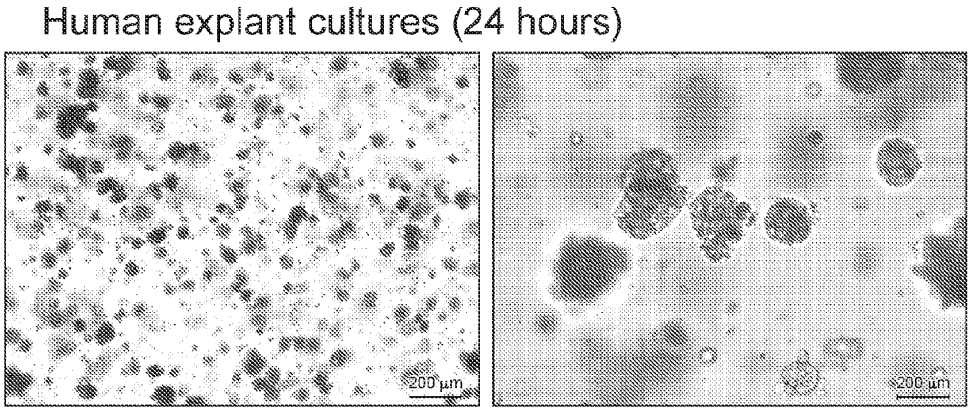


FIG. 11A FIG. 11B

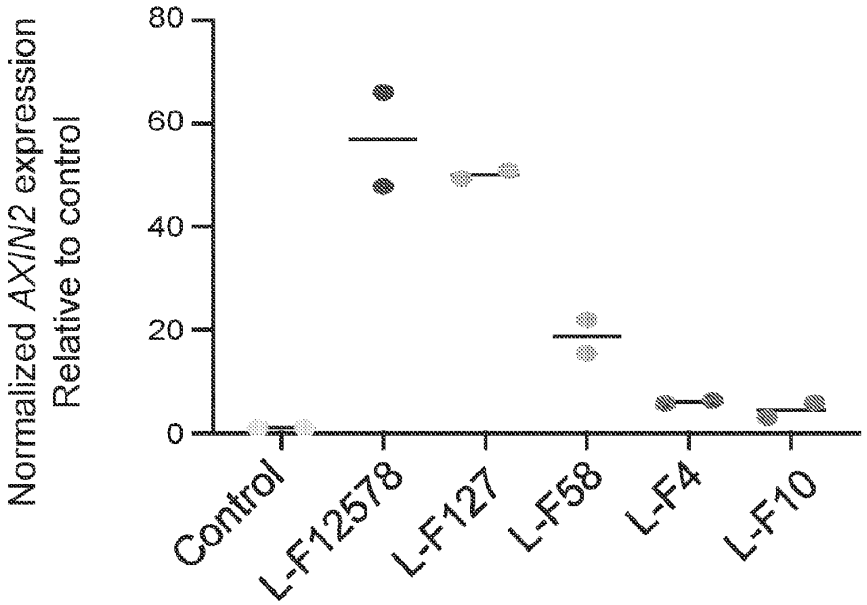


FIG. 12

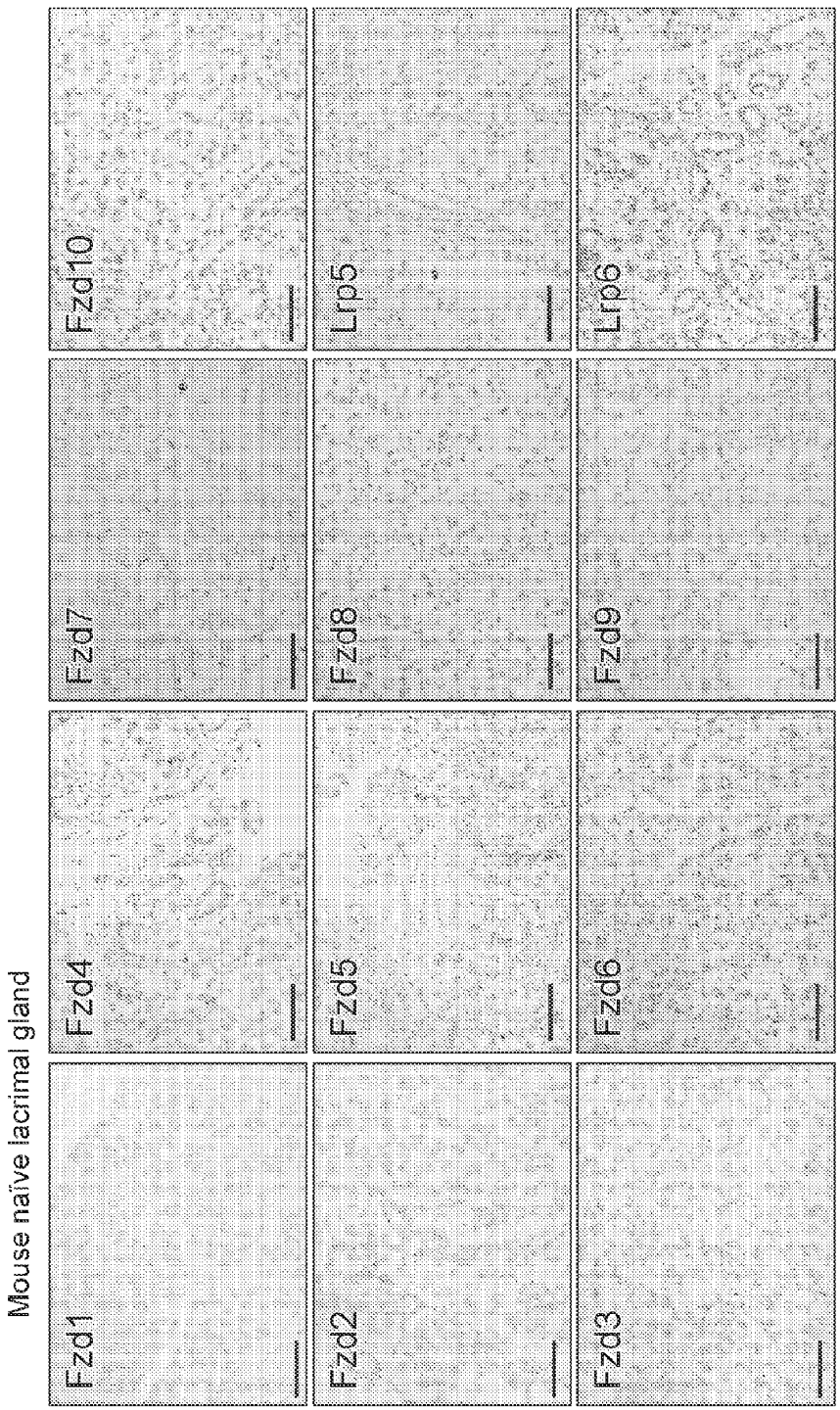

Mouse naïve lacrimal gland
Fzd1
Fzd4
Fzd7
Fzd10
Fzd2
Fzd5
Fzd8
Lrp5
Fzd3
Fzd6
Fzd9
Lrp6

FIG. 13

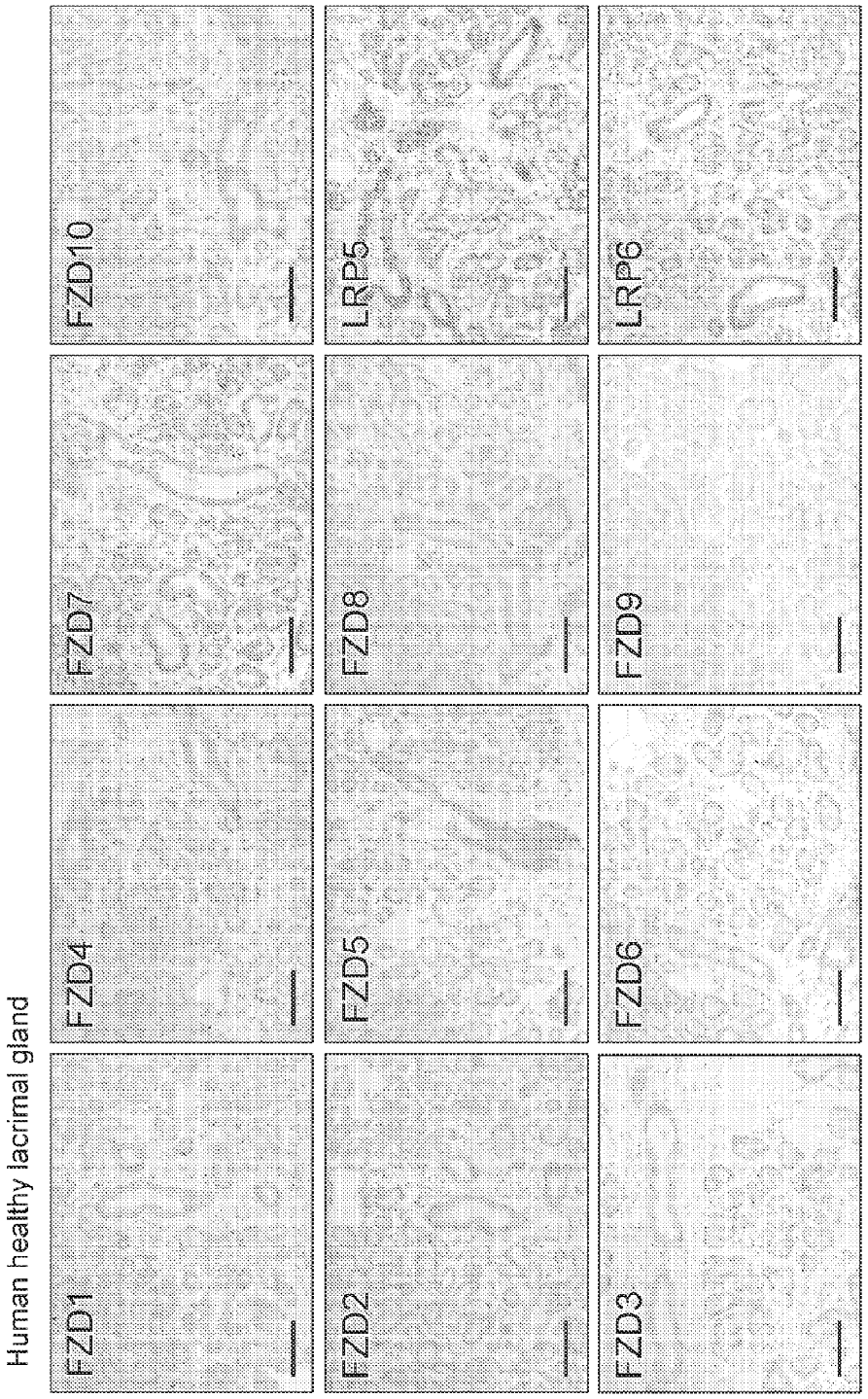

Human healthy lacrimal gland
FZD1
FZD4
FZD7
FZD10
FZD2
FZD5
FZD8
LRP5
FZD3
FZD6
FZD9
LRP6

FIG. 14

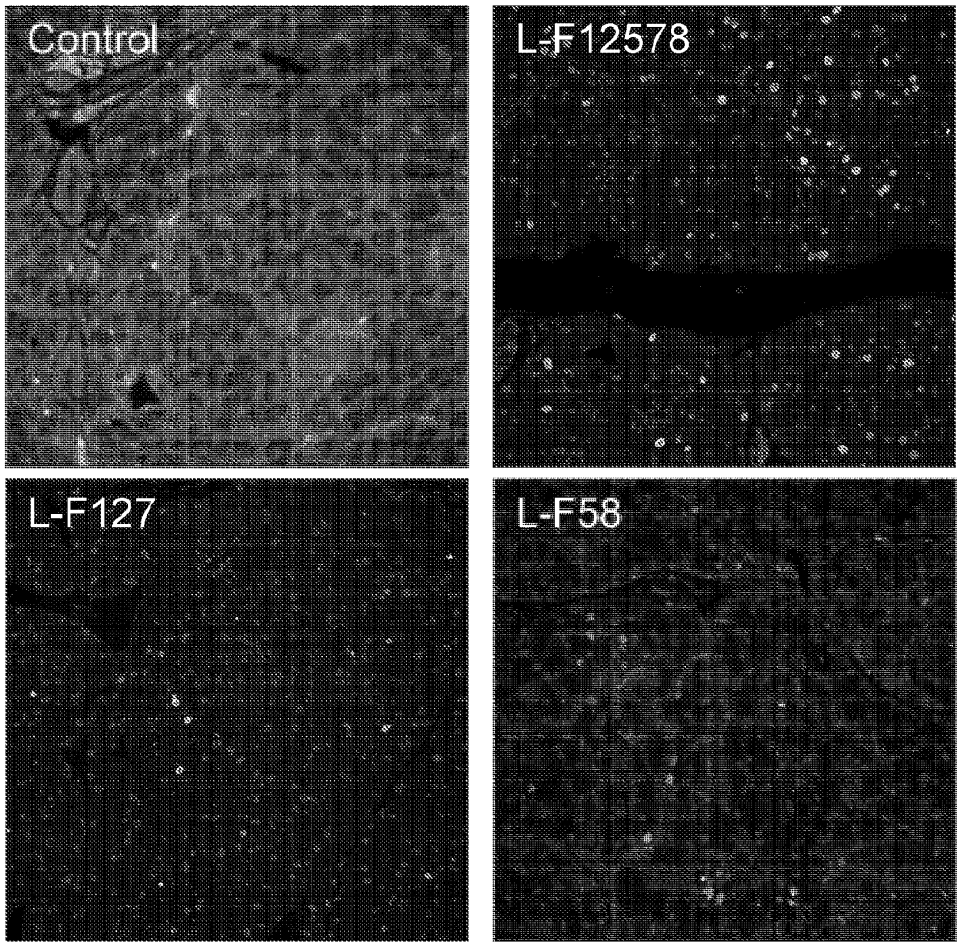


FIG. 15

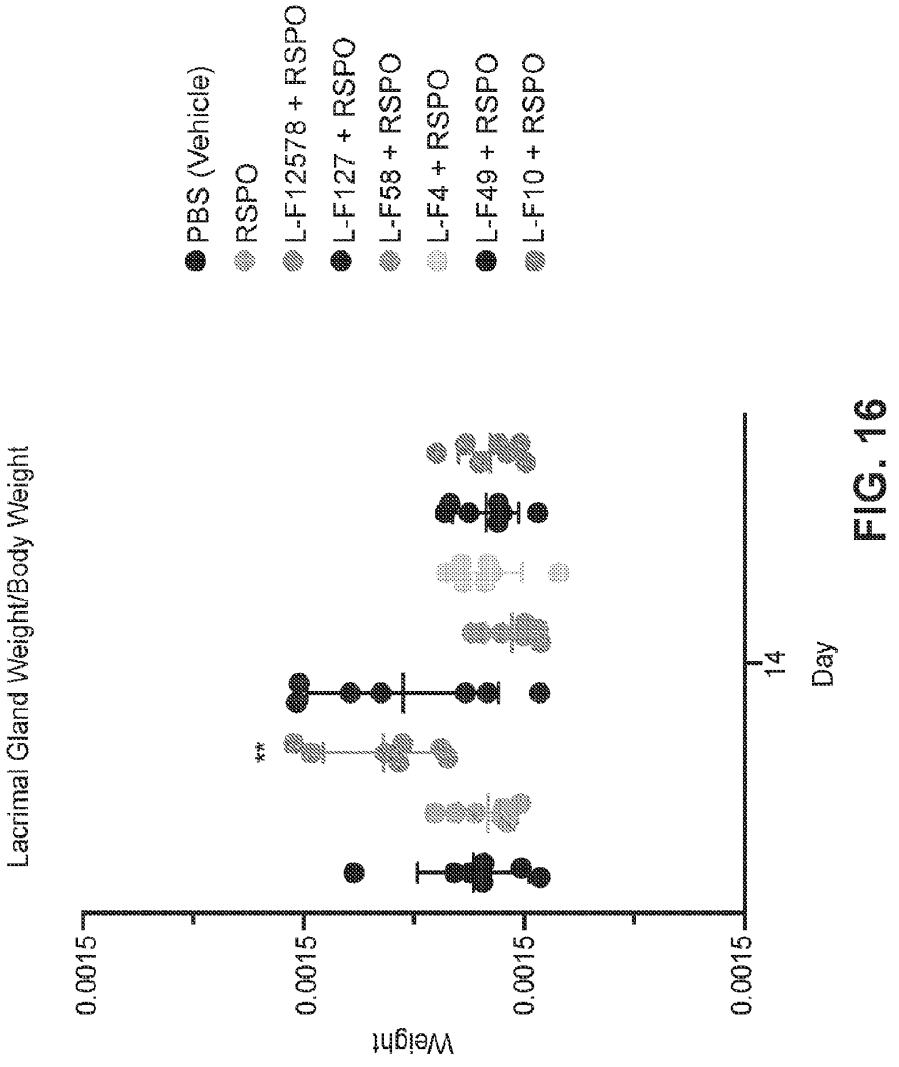

PBS (Vehicle)
RSPO
L-F12578 + RSPO
L-F127 + RSPO
L-F58 + RSPO
L-F4 + RSPO
L-F49 + RSPO
L-F10 + RSPO
Lacrimal Gland Weight/Body Weight
Weight
0.0015
0.0015
0.0015
0.0015
**
14
Day

FIG. 16

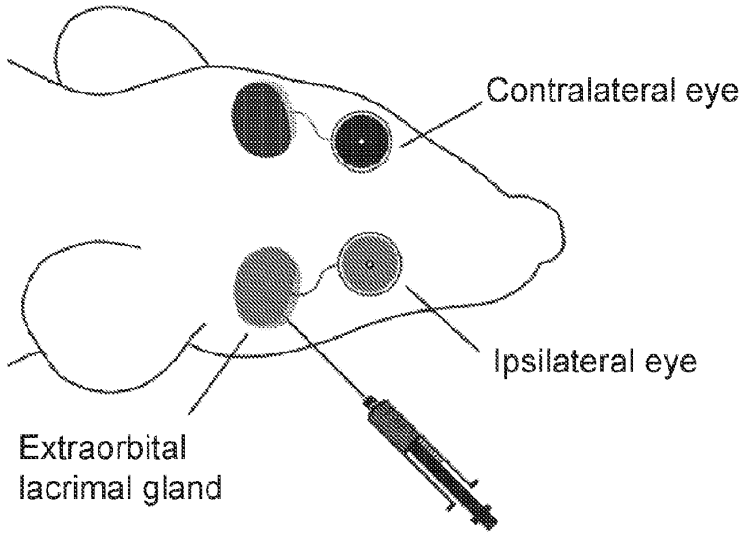


FIG. 17

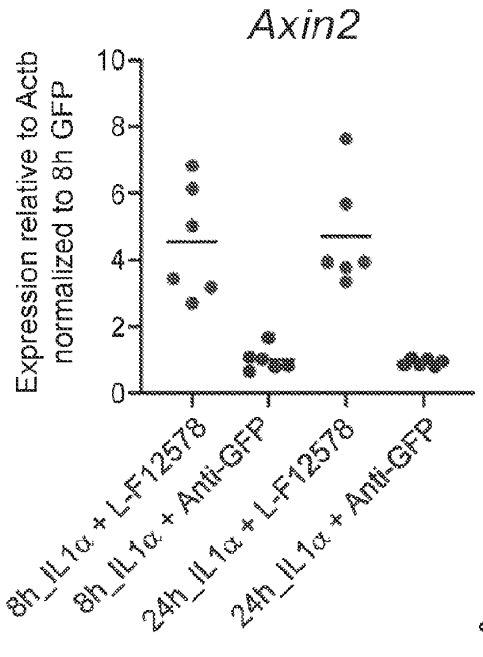


FIG. 18A

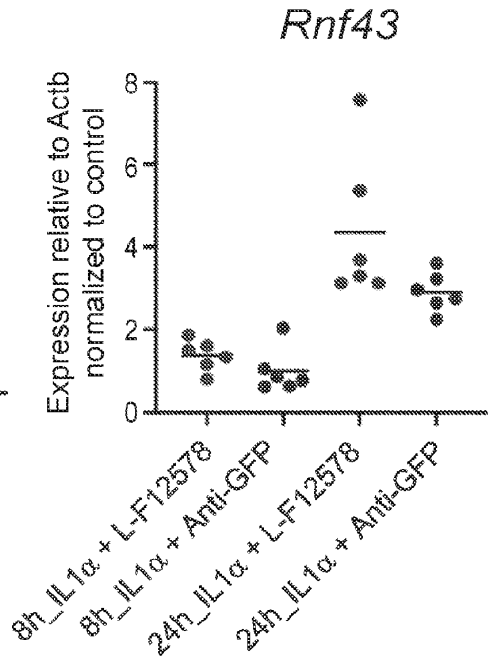


FIG. 18B

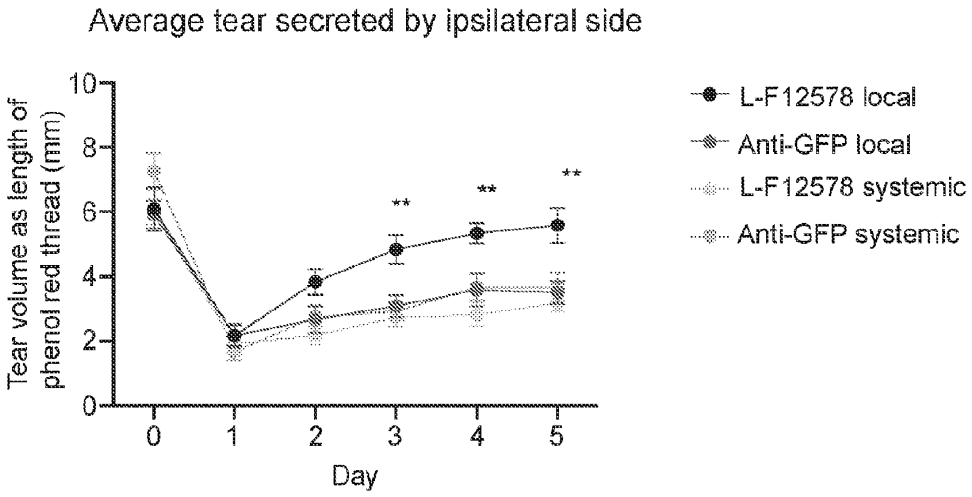

Average tear secreted by ipsilateral side
Tear volume as length of phenol red thread (mm)
10
8
6
4
2
0
0
1
2
3
4
5
Day
**
**
**
L-F12578 local
Anti-GFP local
L-F12578 systemic
Anti-GFP systemic

FIG. 19

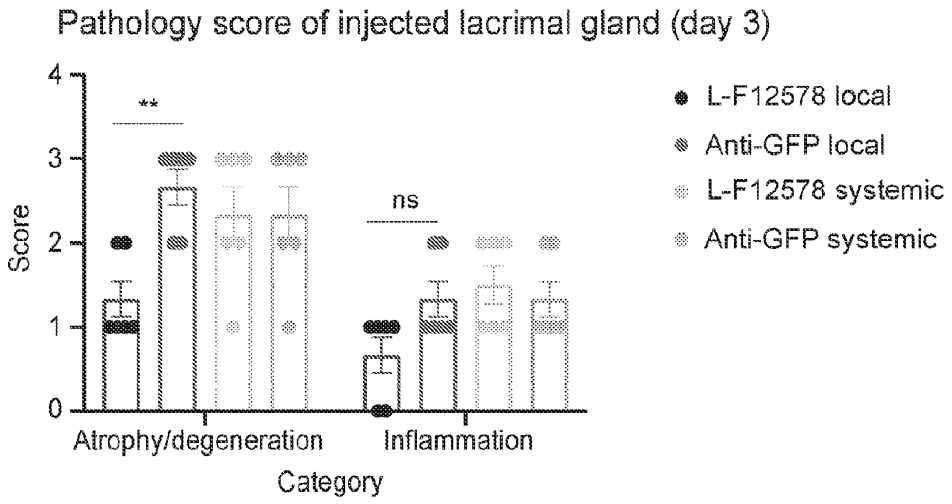
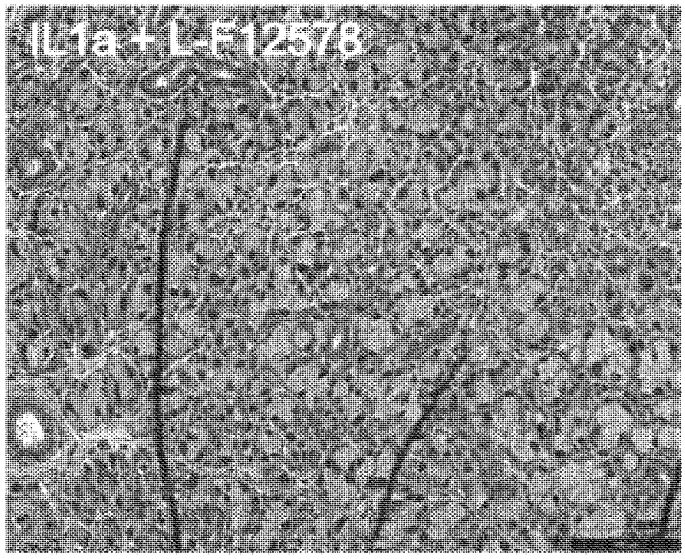

Pathology score of injected lacrimal gland (day 3)
Score
4
3
2
1
0
**
ns
Atrophy/degeneration
Inflammation
Category
L-F12578 local
Anti-GFP local
L-F12578 systemic
Anti-GFP systemic

FIG. 20

FIG. 21A



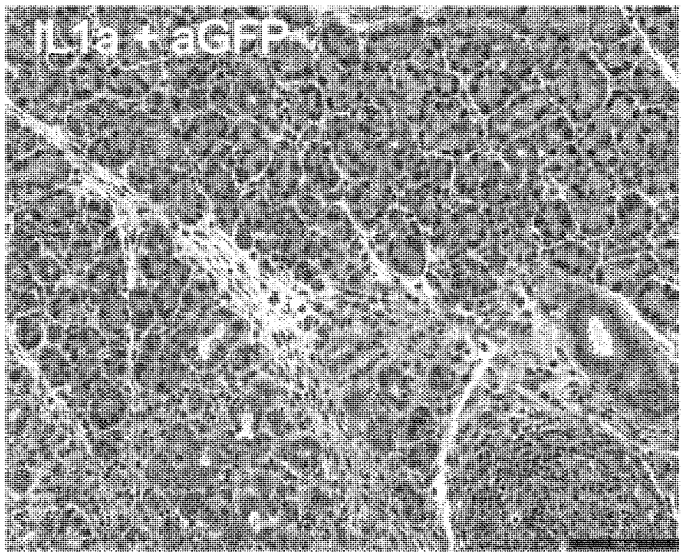


FIG. 21B

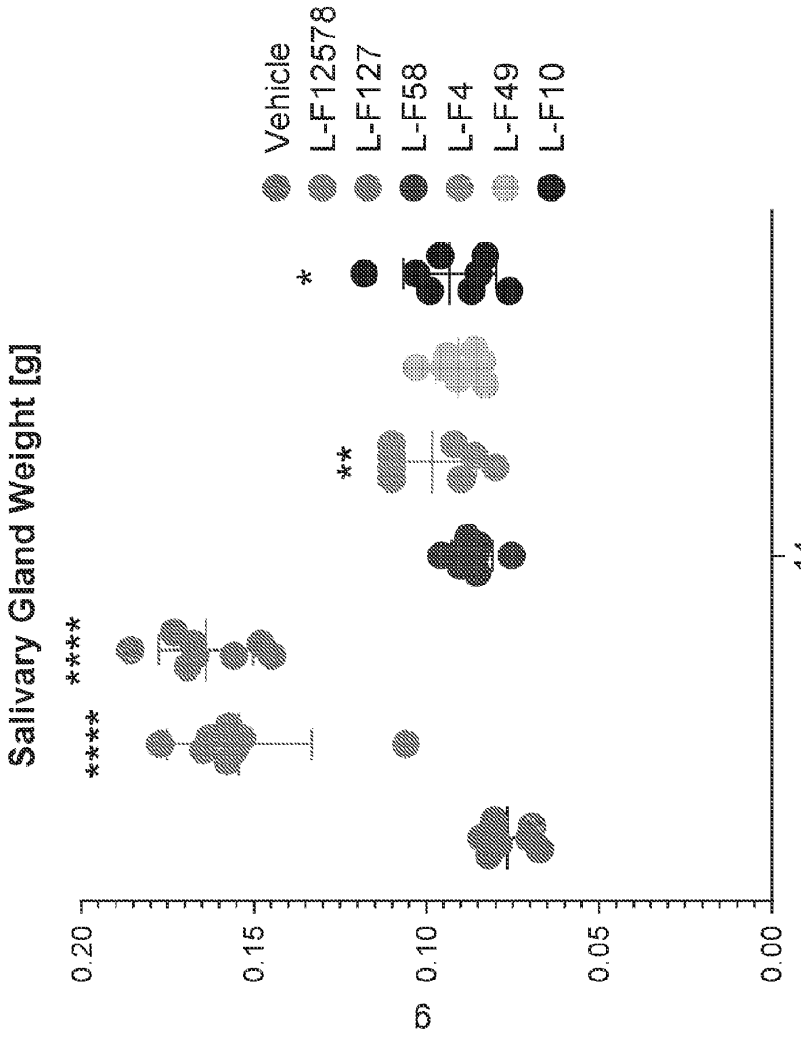

Salivary Gland Weight [g]
0.20
0.15
0.10
0.05
0.00
****
****
*
**
Vehicle
L-F12578
L-F127
L-F58
L-F4
L-F49
L-F10
14
Day

FIG. 22

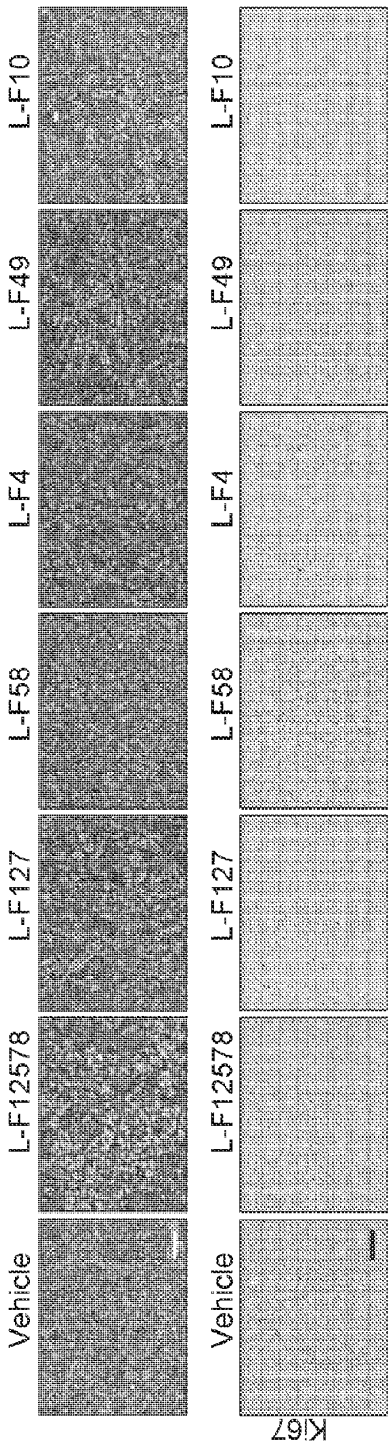

Vehicle
L-F12578
L-F127
L-F58
L-F4
L-F49
L-F10
Vehicle
L-F12578
L-F127
L-F58
L-F4
L-F49
L-F10
Ki67

FIG. 23

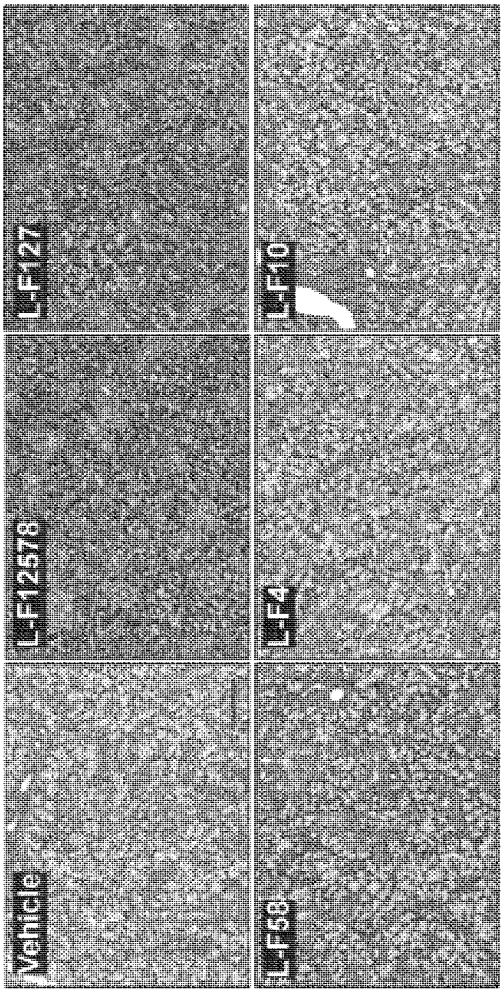


FIG. 24A

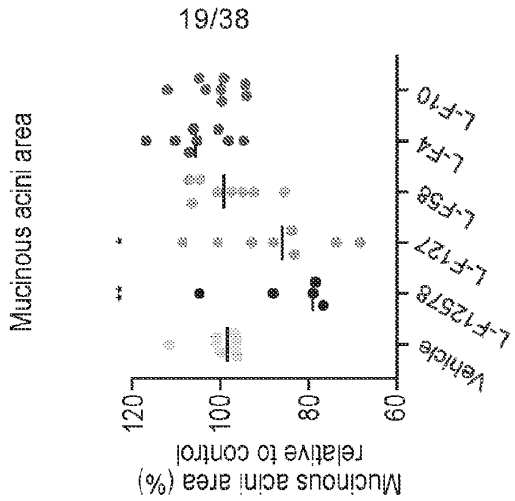


FIG. 24B

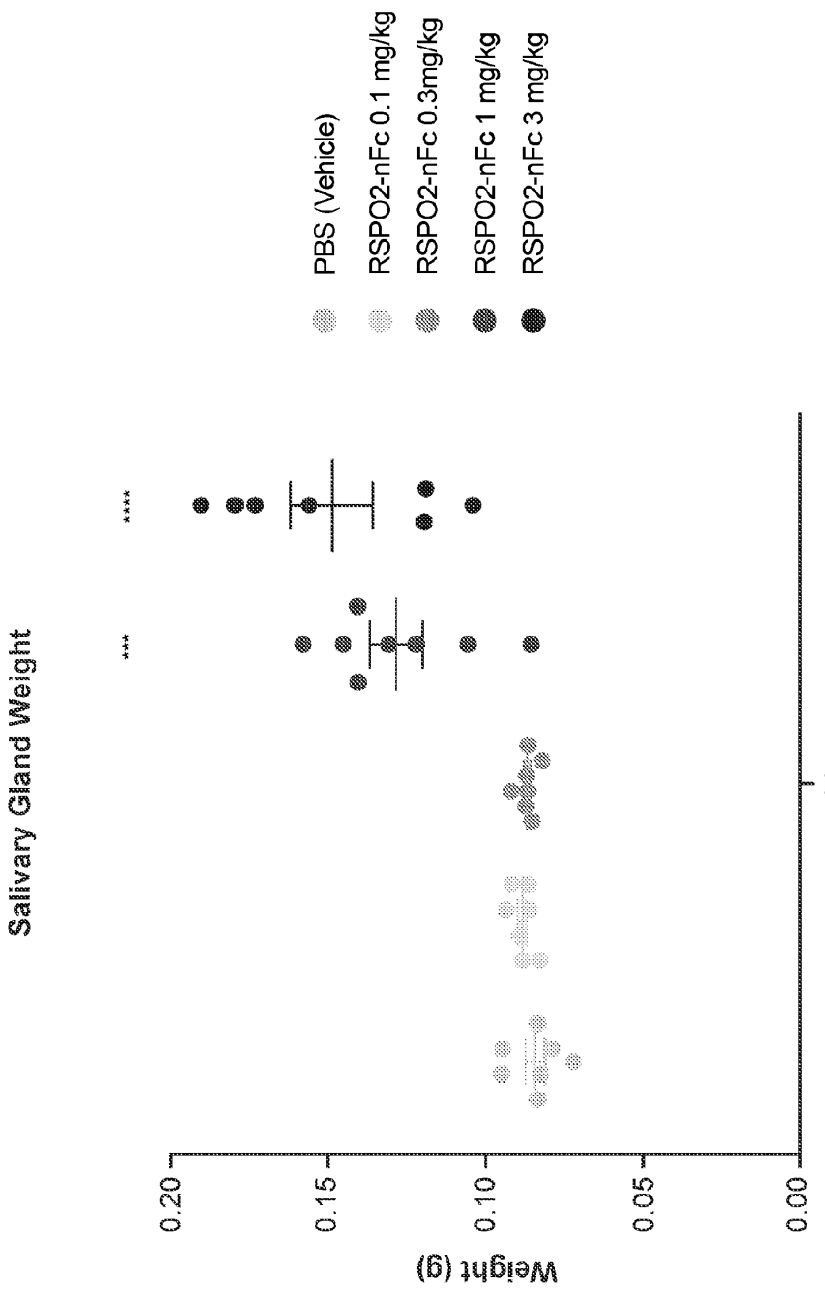

Salivary Gland Weight
PBS (Vehicle)
RSPO2-nFc 0.1 mg/kg
RSPO2-nFc 0.3mg/kg
RSPO2-nFc 1 mg/kg
RSPO2-nFc 3 mg/kg
***
****
0.20
0.15
0.10
0.05
0.00
Weight (g)
14
Day

FIG. 25