The complex world of WNT receptor signalling

Christof Niehrs

Abstract | 30 years after the identification of WNTs, their signal transduction has become increasingly complex, with the discovery of more than 15 receptors and co-receptors in seven protein families. The recent discovery of three receptor classes for the R-spondin family of WNT agonists further adds to this complexity. What emerges is an intricate network of receptors that form higher-order ligand–receptor complexes routing downstream signalling. These are regulated both extracellularly by agonists such as R-spondin and intracellularly by post-translational modifications such as phosphorylation, proteolytic processing and endocytosis.

Embryonic axis
The ensemble of head-to-tail structures of early embryos.

Segmentation
An embryonic process that divides the embryo or parts of it into repetitive segments.

Division of Molecular Embryology, DKFZ-ZMBH Alliance, DKFZ, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, and Institute of Molecular Biology, 55128 Mainz, Germany.
e-mail: C.Niehrs@imb-mainz.de
doi:10.1038/nrm3470
Published 15 November 2012

This year marks the thirtieth anniversary of the discovery of the *Int1* proto-oncogene, now known as *Wnt1*, as the locus associated with mouse mammary tumour virus-driven tumorigenesis[1]. Since then, research in WNT signalling has become a field in its own right. These secreted Cys-rich growth factors play key parts as intercellular signalling molecules in development and in disease. Very fittingly, this year saw the publication of the long-awaited structure not only of the first WNT protein but also of the ligand in complex with its main seven transmembrane receptor Frizzled[2].

Of the multitude of developmental processes that involve WNT signalling, some of the more prominent ones are primary embryonic axis formation, segmentation and appendage patterning in *Drosophila melanogaster*, organogenesis from worms to mice, as well as stem cell proliferation[3–7]. WNT signalling is also implicated in various diseases, including colon cancer and melanoma, as well as neurodegenerative diseases[8]. For example, more than 90% of colorectal cancers involve hyperactivated β-catenin-dependent signalling[8].

Over the past 10 years, our understanding of WNT signalling has increased enormously by the identification of cell surface receptors for WNTs and by deciphering a network of intracellular transduction components. The WNT signalling network is of bewildering complexity, containing numerous components and being subject to many regulatory steps and cross-talk mechanisms. WNT receptors and co-receptors include members of the Frizzled[9], low-density lipoprotein receptor-related protein 6 (LRP6)[10], receptor Tyr kinase-like orphan receptor (ROR)[11], protein Tyr kinase 7 (PTK7)[12], receptor Tyr kinase (RYK)[13], muscle skeletal receptor Tyr kinase (MUSK)[14] and proteoglycan families[15], and we are beginning to understand how they interact and which pathways they engage. These receptors are regulated intracellularly by protein phosphorylation and extracellularly by various secreted antagonists, including Cerberus, Dickopf-related protein 1 (DKK1), secreted Frizzled-related protein (SFRP), WNT inhibitory factor (WIF), Sclerostin (and its homologue Wise)[16] and two classes of WNT agonists, the R-spondin family and Norrin. Notably, R-spondins have important roles in development and cancer and act as powerful stem cell growth factors[17]. However, the identity of R-spondin receptors and their mechanism of action have remained enigmatic. In this respect, exciting progress has been made recently with the discovery of three classes of transmembrane proteins that interact with R-spondins: syndecans[18], Leu-rich repeat-containing G protein-coupled receptors (LGRs)[19–21] and transmembrane E3 ubiquitin ligases[22,23].

In this Review, I focus on intracellular and extracellular WNT receptor regulation, including the recent progress that has been made in WNT–R-spondin signalling, but excluding extracellular WNT antagonists, which have been reviewed elsewhere[16,24]. Reviews summarizing other aspects of WNT signalling mechanisms, including comprehensive overviews of WNT signal transduction and regulation of β-catenin-dependent and -independent signalling[15,25–27], WNT trafficking[28], WNT signalling, cell cycle regulation[29] and transcriptional regulation[30], are available.

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Overview of WNT signalling

WNTs are evolutionarily conserved secreted Cys-rich proteins, and in humans there are 19 genes that encode WNTs[15]. They couple to various receptors and thereby activate different downstream pathways. On the basis of early studies, these pathways have been classified as either canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) signalling pathways. However, this classification can only serve as a rough guide, as within canonical and non-canonical WNT signalling various sub-branches are used in different cellular contexts[15,25,26].

Although several WNTs preferentially activate either β-catenin-dependent or β-catenin-independent pathways, the activity of WNTs depends on the cellular context and the receptor, and thus WNTs cannot be rigorously subdivided according to the pathway they induce. This is because there are more than 15 different WNT receptors and co-receptors, and the particular combination of these together with a given WNT determines the downstream pathway (FIGS 1,2). This combinatorial code, one of the key unsolved questions in WNT signalling, is beginning to be elucidated. Despite this complexity, it is a useful approximation that, for

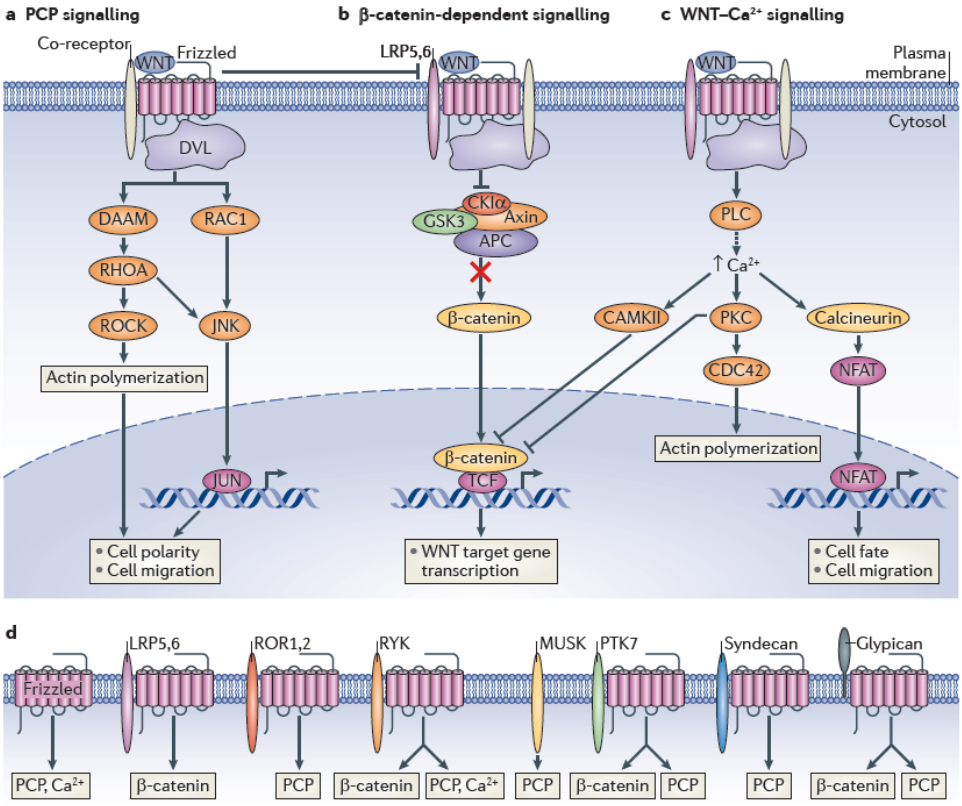

Figure 1 | **WNT signalling pathways.** Simplified schemes showing the main WNT pathways directed by specific WNT, Frizzled and WNT co-receptor interactions. **a** | Planar cell polarity (PCP) signalling triggers activation of the small GTPases RHOA and RAC1, which in turn activate RHO kinase (ROCK) and JUN-N-terminal kinase (JNK), respectively, leading to actin polymerization and microtubule stabilization. This pathway is prominently involved in the regulation of cell polarity, cell motility and morphogenetic movements. **b** | Under steady-state conditions, glycogen synthase kinase 3 (GSK3) phosphorylates β-catenin, which triggers its degradation. However, in the presence of WNT ligand, the destruction complex (comprising GSK3, casein kinase Iα (CKIα), Axin and adenomatosis polyposis coli (APC)) is recruited to the WNT–receptor complex and inactivated. This allows β-catenin to accumulate and translocate to the nucleus, where it activates the transcription of target genes under the control of T cell factor (TCF), among others. **c** | The WNT–Ca^{2+} pathway activates Ca^{2+}- and calmodulin-dependent kinase II (CAMKII), protein kinase C (PKC) and calcineurin. Calcineurin activates nuclear factor of activated T cells (NFAT), which regulates the transcription of genes controlling cell fate and cell migration. PCP and Ca^{2+} pathways antagonize β-catenin signalling at various levels. **d** | Major pathways used by WNT receptors and co-receptors. Only the three best-characterized WNT pathways are shown: the β-catenin-dependent pathway (β-catenin); the PCP pathway; and the WNT–Ca^{2+} pathway (Ca^{2+}). For other receptor downstream responses see main text. DAAM, DVL-associated activator of morphogenesis 1; DVL, Dishevelled; LRP, low-density lipoprotein receptor-related protein; MUSK, muscle skeletal receptor Tyr kinase; PLC, phospholipase C; PTK7, protein Tyr kinase 7; ROR, receptor Tyr kinase-like orphan receptor; RYK, receptor Tyr kinase.

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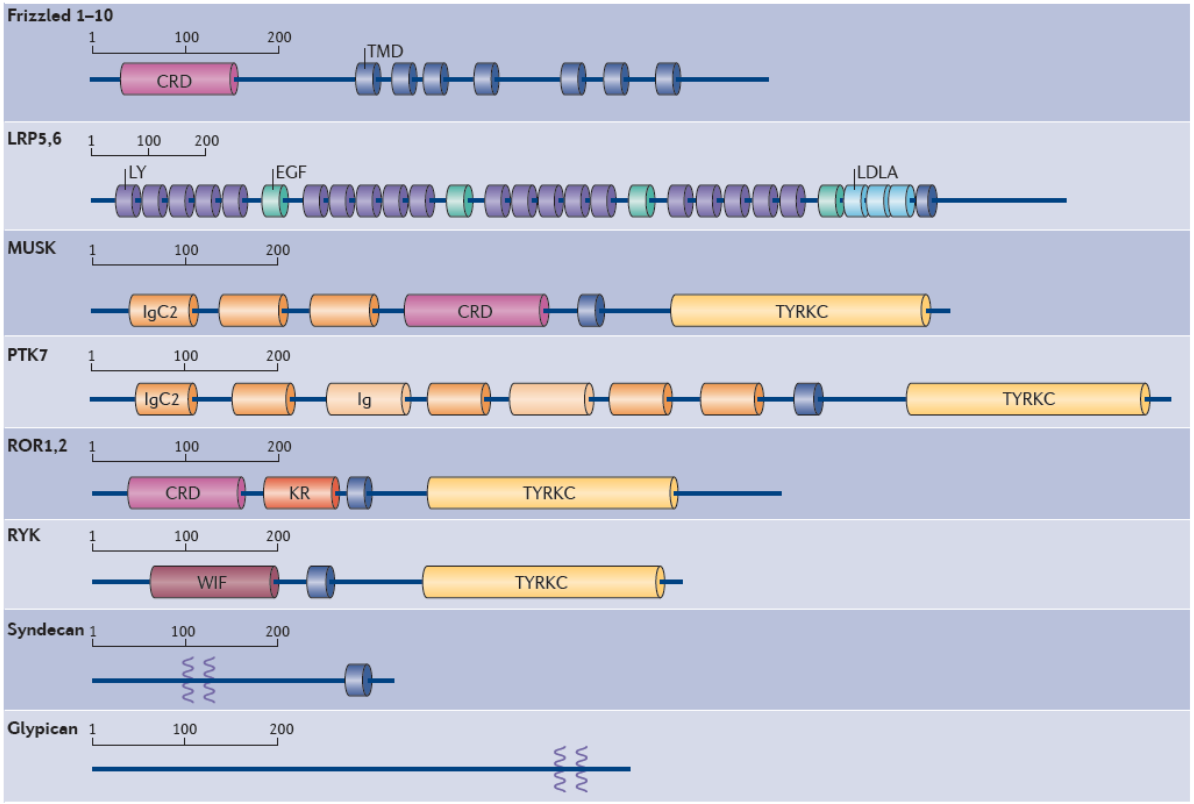

Figure 2 | **WNT receptors and co-receptors.** Domain structures of WNT receptors and co-receptors according to the SMART database. Syndecans and glypicans include proteoglycan chains (purple). Extracellular domains to the left, intracellular to the right, wherever applicable. CRD, Cys-rich domain; EGF, epidermal growth factor-like domain; Ig, immunoglobulin; KR, kringle domain; LDLA, low-density lipoprotein receptor domain class A; LRP, low-density lipoprotein receptor-related protein; LY, low-density lipoprotein receptor YWTD domain; MUSK, muscle skeletal receptor Tyr kinase; PTK7, protein Tyr kinase 7; RYK, receptor Tyr kinase; ROR, receptor Tyr kinase-like orphan receptor; TMD, transmembrane domain; TYRKC, Tyr kinase catalytic domain; WIF, WNT-inhibitory factor-1 like domain.

example, WNT1, WNT3A and WNT8 are more commonly encountered in β-catenin-dependent signalling, and WNT5A and WNT11 are predominantly involved in β-catenin-independent signalling[15]. As another rule, Frizzled proteins act as common receptors for both the β-catenin-dependent and β-catenin-independent pathways, and the use of LRP5 and LRP6 or ROR1 and ROR2 determines β-catenin-dependent and β-catenin-independent pathways, respectively.

β-catenin-dependent signalling. The β-catenin-dependent pathway is the best-characterized pathway and is triggered by the interaction of WNT with Frizzled and LRP5 or LRP6 (FIG. 1b). The molecular cascade involved in β-catenin-dependent WNT signalling leads to the inhibition of glycogen synthase kinase 3 (GSK3), which regulates various substrates. One key substrate of GSK3 is the transcriptional co-activator β-catenin, which is inactivated by GSK3-mediated phosphorylation and targeted for proteasomal degradation in unstimulated cells. Inactivation of β-catenin involves a 'destruction complex' that in addition to GSK3 comprises adenomatosis polyposis coli (APC), Axin and casein kinase Iα (CKIα). Upon WNT activation, cytoplasmic β-catenin is stabilized and enters the nucleus, where it associates with transcription factors, notably TCF (T cell factor) and LEF (lymphoid enhancer-binding factor), to regulate the transcription of target genes. This pathway is prominently involved in regulating cell differentiation and proliferation[4,6,31].

β-catenin is a key target of the canonical WNT pathway, and it mediates many cellular processes in response to WNT. In *D. melanogaster* and *Xenopus laevis* early embryos, loss of β-catenin phenocopies loss of Wingless and wnt11, respectively[32,33], which indicates that these proteins transduce predominantly through β-catenin in these contexts. However, it needs to be appreciated that GSK3 regulates several other substrates downstream of WNT signalling, as has been elucidated especially in mammalian cells. Such GSK3 substrates are involved in cytoskeleton and cell cycle regulation, for example cyclins and the translation initiation factor eIF2B[29].

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Recent data indicate that GSK3 mediates the degradation of hundreds of proteins in a WNT-dependent manner[34,35]. This is because GSK3-phosphorylated protein motifs are abundant and once phosphorylated can be recognized by E3 polyubiquitin ligases, which target proteins for proteasomal degradation[34,35].

β-catenin-independent WNT signalling. β-catenin-independent WNT signalling encompasses those pathways that do not use the β-catenin–TCF or β-catenin–LEF module but instead use other modes of downstream signalling, some of which elicit a transcriptional response. There is now a confusing array of such non-canonical WNT pathways, classified according to the WNT receptors or co-receptors they use and which downstream effectors they are coupled to. It is still controversial to what extend these pathways may be at least partially overlapping. For example, there is now good evidence that in vertebrates the planar cell polarity (PCP) and WNT5A–ROR signalling pathways overlap substantially[36–38].

The best-characterized β-catenin-independent pathway is the PCP pathway (FIG. 1a). In PCP signalling, Frizzled receptors activate a cascade that involves the small GTPases RAC1 and RHOA and JUN-N-terminal kinase (JNK). The downstream output of this pathway can lead to changes in cytoskeleton and cell polarity via small GTPases and/or transcriptional activation of JNK-dependent transcription factors, for example activating transcription factor 2 (ATF2), with concomitant activation of their target genes[15,26]. The PCP pathway is prominently involved in regulating cell polarity in morphogenetic processes. For example, in vertebrates PCP signalling regulates cell movement during gastrulation, neural tube closure and the orientation of stereocilia in the inner ear[15,26]. PCP and β-catenin-dependent WNT signalling are well known to antagonize each other, and inhibiting one will typically upregulate the other. This antagonistic crosstalk occurs, in part, at the ligand–receptor level. For example, WNT5A, which preferentially activates PCP signalling, competes for and inhibits binding of WNT3A to Frizzled 2, thereby suppressing the β-catenin-dependent pathway[39]. PCP signalling does not require LRP5 or LRP6 but instead uses ROR and PTK7 as co-receptors. It is unclear whether ROR and PTK7 are alternative Frizzled co-receptors in different cells, or whether they interact with each other and Frizzled. Moreover, unlike vertebrates, in flies it is still unclear whether a WNT ligand is involved in the otherwise well-characterized PCP signalling pathway.

The second β-catenin-independent pathway that has been described is the WNT–Ca^{2+} pathway (FIG. 1c), which was initially identified in *X. laevis* and zebrafish[40] embryos. In this case, WNTs trigger Frizzled-mediated activation of heterotrimeric G proteins. This activates phospholipase C (PLC), which in turn stimulates diacylglycerol and inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) production. $Ins(1,4,5)P_3$ triggers Ca^{2+} release from intracellular stores and activation of effectors such as Ca^{2+}- and calmodulin-dependent kinase II (CAMKII), calcineurin and protein kinase C (PKC), which activate the transcriptional regulator nuclear factor associated with T cells (NFAT)[27]. This pathway is involved in cancer, inflammation and neurodegeneration[27].

Besides these main β-catenin-independent pathways, there are additional tissue-specific downstream events triggered by WNTs in combination with Frizzled or specific co-receptors that do not fall into these categories and are discussed below.

WNT–Frizzled interaction

WNTs are hydrophobic owing to lipid modification[41] with palmitoleic acid on a conserved Ser residue[42] (Ser209 in WNT3A; Ser187 in *X. laevis* wnt8). Acylation is necessary for both intracellular trafficking of WNTs and their full activity when secreted. Because of their hydrophobicity, WNTs are difficult to purify without detergent[41] and are unlikely to physiologically travel as 'naked' proteins. Consistent with this, in *D. melanogaster* Wingless co-purified with lipoprotein particles, and larvae with a reduced number of lipoprotein particles showed reduced Wingless signalling[43]. Similarly, in mammalian fibroblasts WNT3A can be released from high-density lipoprotein particles in the culture medium[44]. Association of WNTs with lipoprotein particles may aid their extracellular diffusion, but whether this interaction influences receptor recognition is unclear[28].

Frizzled proteins are the principal WNT receptors and contain a large extracellular Cys-rich domain (CRD), which mediates WNT binding. The structure of *X. laevis* wnt8 in complex with the CRD of mouse Frizzled 8 has recently been solved and yielded important new insights into WNT biochemistry[2] (FIG. 3). First, it revealed that all Cys residues of wnt8 are engaged in intramolecular disulphide bonds and thus are unlikely to be post-translationally modified. Second, the three-dimensional structure confirmed that wnt8 binds Frizzled 8 as a monomer. Third, it revealed a two-domain wnt8 structure that is unrelated to known protein folds, with two finger-like domains grasping the Frizzled 8 CRD at two binding sites. The amino-terminal domain of wnt8 (also termed 'lipid thumb') is dominated by the palmitoleic lipid projecting into a groove in the Frizzled 8 CRD. The wnt8 lipid thumb interacts with amino acid residues that are conserved among WNT–Frizzled family members. This may explain the promiscuous interactions of many WNT–Frizzled proteins. The other wnt8 domain, a carboxy-terminal 'index finger', engages in hydrophobic contacts with amino acid residues that are highly conserved in all WNTs. The index finger interacts with a small depression of the Frizzled 8 CRD, which harbours some residues that are not conserved among other Frizzled CRDs. Thus, it is possible that the specificity in WNT–Frizzled binding arises from the interaction of the WNT index finger with this surface domain in Frizzled. The structure of wnt8 in complex with the Frizzled 8 CRD may explain how WNTs can form ternary complexes by binding simultaneously to various co-receptors, such as LRPs, RORs and PTK7, because the remaining accessible surface patch on wnt8 is now an obvious candidate for such multivalent interactions.

Planar cell polarity
(PCP). Cell polarity within the plane of an epithelial sheet.

Gastrulation
The morphogenetic process whereby embryos acquire the germ layers.

Stereocilia
Tapered, finger-like projections from hair cells of the inner ear that respond to mechanical displacement with alterations in membrane potential and thereby mediate sensory transduction.

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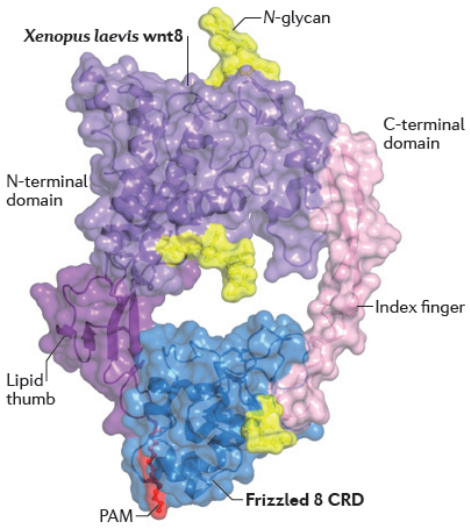

Figure 3 | **Crystal structure of the wnt8–Frizzled 8 CRD complex.** Overall structure of *Xenopus laevis* wnt8 bound to the Frizzled 8 Cys-rich domain (CRD) shown in 'face on' representation. *X. laevis* wnt8 core is coloured in purple, the amino-terminal lipid thumb domain in deep purple, the carboxy-terminal index finger in light pink and palmitoleic acid (PAM) in red. The Frizzled 8 CRD is shown in blue and *N*-glycans in yellow. This figure is reproduced, with permission, from REF. 2 © (2012) AAAS.

WNT receptors and their regulation

Frizzled proteins are widespread high-affinity WNT receptors that use co-receptors as a major regulatory determinant to direct downstream signalling to distinct signalling branches (FIG. 1d). Whether they can also function without co-receptors is unknown and difficult to prove. In general, co-receptors are cell surface proteins that associate with a ligand and its primary receptor to enhance complex formation and downstream signalling. In β-catenin-dependent signalling, WNT–Frizzled form a ternary complex with LRP5 or LRP6, whereas in PCP signalling they engage ROR or PTK7 (REFS 38,39,45–47). Frizzled can also interact with RYK to activate β-catenin-dependent, PCP and WNT–Ca^{2+} downstream pathways (see below).

In practice it can be difficult to distinguish between receptor and co-receptor function for a given WNT-binding cell surface protein. For example, ROR2 binds to a WNT–Frizzled complex and promotes downstream PCP signalling[38,39], indicating co-receptor function. However, ROR binds WNT directly and can trigger WNT signalling[37,45], and therefore it is likely that ROR signals independently of Frizzled and hence may also be considered a receptor. Conversely, there is yet no evidence that RYK and MUSK bind Frizzled, which may define them as additional WNT receptors in certain contexts rather than Frizzled co-receptors. Hence, for simplicity, here Frizzled is discussed as the primary WNT receptor and other WNT-interacting transmembrane proteins as co-receptors (FIG. 2).

Frizzled and WNT co-receptors are tightly regulated to prevent excessive signalling, which otherwise could lead to misregulation of cell differentiation and/or proliferation. There are recurring themes of how Frizzled and WNT co-receptors are intracellularly regulated, either positively or negatively. This includes receptor phosphorylation (in the case of Frizzled, LRP6, ROR and MUSK), ubiquitylation accompanied by cell surface removal (for Frizzled and RYK), proteolytic release of the cytoplasmic domain (Frizzled, LRP6 and RYK) or extracellular domain release by cleavage of a lipid anchor (TABLE 1).

The WNT receptor Frizzled. The Frizzled class comprises 10 family members in humans, and Frizzled proteins were the first WNT receptors identified[48]. They are the most widespread WNT receptors and transmit β-catenin-dependent and β-catenin-independent signalling. Frizzled proteins contain a large extracellular CRD, which is conserved across the receptor family and mediates high-affinity interactions with WNT proteins[2,49] (see above). A related CRD domain is also found in the otherwise unrelated WNT co-receptors ROR and MUSK (see below). However, Frizzled might have additional low-affinity WNT-binding sites[50]. Like G protein-coupled receptors (GPCRs), Frizzled receptors are transmembrane proteins, and there is now considerable evidence that Frizzled receptors can dimerize and are coupled to heterotrimeric G proteins[51], for example to recruit activating kinases to the receptor complex[52].

Frizzled 4 can be regulated by the secreted protein Norrin, binding of which activates β-catenin-dependent signalling in an LRP5-, LRP6- and WNT-independent manner[53]. Norrin binds specifically to the Frizzled 4 CRD and does not detectably bind to the 14 other mammalian Frizzled and secreted Frizzled-related protein CRDs[54]. Norrin multimers cooperate with LRP5 and the four-pass transmembrane protein tetraspanin 12 to promote Frizzled 4 oligomerization, thereby activating the β-catenin-dependent pathway, possibly by initiating WNT receptor endocytosis[55]. Whether Norrin signalling requires LRP5 or LRP6 is unknown.

Further regulation of Frizzled receptors can occur through intracellular binding to cytoplasmic partners, primarily through the Frizzled cytoplasmic tail or the intracellular domain (ICD). The key cytoplasmic partner is the scaffold protein Dishevelled (DVL), which interacts with most WNT co-receptors and transduces all major WNT subpathways and as such represents an almost universal molecule downstream of WNT receptors[15]. Frizzled engages DVL proteins through their conserved KTXXXW motif[15]. Upon ligand binding, DVL proteins are recruited to Frizzled receptors at the plasma membrane and co-recruit cytoplasmic transducers, such as Axin, CK1 and GSK3 binding protein (GBP), presumably along with their partners, to promote β-catenin-dependent signalling. Importantly, DVL itself forms oligomers[56], which may cluster WNT–Frizzled receptor complexes and promote endocytosis and downstream signalling[57]. In PCP signalling, DVL binds to proteins such as PKC, atypical PKC (aPKC), DVL-associated activator of morphogenesis 1 (DAAM1),

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Table 1 | **Regulatory post-translational modifications in WNT receptor regulation**

Receptor	Modifying enzyme	Modification site	Modification	Trigger and regulation	Role in WNT signalling	Refs
Frizzled	aPKC	*Drosophila melanogaster* Frizzled 1: Ser554, Ser560 *D. melanogaster* Frizzled 3: Ser576	Phosphorylation	Unknown	Inhibits PCP signalling	59,60
	Unknown ADAM	*D. melanogaster* Frizzled 2: ICD	Carboxy-terminal proteolysis	Unknown	Promotes signalling and synaptogenesis	63
	UBPY (also known as USP8)	Unknown	Deubiquitylation	Unknown	Activates β-catenin-dependent signalling	61
	ZNRF3, RNF43	Unknown	Ubiquitylation	Unknown	Inhibits Frizzled and so β-catenin-dependent and PCP signalling	22,23
LRP6	GSK3	PPPSP	Phosphorylation	WNT	Primes for β-catenin-dependent signalling	65
	GRK5, GRK6	PPPSP	Phosphorylation	WNT	Primes for β-catenin-dependent signalling	67
	PKA	PPPSP	Phosphorylation	Parathyroid hormone	Primes for β-catenin-dependent signalling	132
	MAPK	PPPSP	Phosphorylation	Phorbol ester, FGF	Primes for β-catenin-dependent signalling	69
	CDK14, cyclin Y	PPPSP	Phosphorylation	G2 to M phase entry	Primes for β-catenin-dependent signalling	70
	CKIγ	Ser residues adjacent to PPPSP; Trp1479	Phosphorylation	WNT	Activates β-catenin-dependent signalling	65,71
	CKII	Ser1579	Phosphorylation	WNT	Inhibits β-catenin-dependent signalling	76
	C1q	NA	Proteolysis	Unknown	Activates β-catenin-dependent signalling	77
ROR2	ROR2	Ser-Thr-Pro domain	Phosphorylation	CKIε	Unknown	86
	CKIε	Ser-Thr-Pro domain	Phosphorylation	Unknown	Activates signalling	86; but see REF. 84
	GSK3	Unknown	Phosphorylation	WNT5A	Activates PCP signalling	38,84
	TAK1	Ser-Thr-Pro domain	Phosphorylation	WNT-dependent?	Inhibits β-catenin-dependent signalling	85
RYK	MIB1	Unknown	Ubiquitylation	Unknown	Activates β-catenin-dependent signalling	94
	γ-secretase	ICD	Proteolysis	Unknown	Activates downstream transcription	62
MUSK	MUSK	Unknown	Autophosphorylation	WNT9, WNT11	Unknown	133
Glypican	Notum	Lipid anchor	Lipid anchor cleavage	Unknown	Inhibits β-catenin-dependent signalling	107,108

ADAM, a disintegrin and metalloprotease; aPKC, atypical PKC; C1q, complement component 1q; CDK14, cyclin-dependent kinase 14; CK, casein kinase; FGF, fibroblast growth factor; GRK, G protein-coupled receptor kinase; GSK3, glycogen synthase kinase 3; ICD, intracellular domain; LRP6, low-density lipoprotein receptor-related protein 6; MAPK, mitogen-activated protein kinase; MIB1, Mind bomb 1; MUSK, muscle skeletal receptor Tyr kinase; NA, not applicable; PCP, planar cell polarity; PKA, protein kinase A; PPPSP, Pro-Pro-Pro-Ser/Thr-Pro; RNF43, RING finger 43; ROR, receptor Tyr kinase-like orphan receptor; RYK, receptor Tyr kinase; TAK1, TGFβ activated protein kinase 1; UBPY, ubiquitin C-terminal hydrolase; ZNRF3, zinc and RING finger 3.

DVL-associating protein with a high frequency of Leu residues (DAPLE) and partitioning defective 6 (PAR6), which are important for the regulation of small GTPases such as RHO and RAC and, consequently, the cytoskeleton and cell polarity[58].

Frizzled receptors can be further regulated by post-translational modifications such as phosphorylation. In *D. melanogaster*, aPKC-mediated Frizzled 1 phosphorylation at its ICD downregulates PCP signalling during eye development in cells in which signalling should not occur[59]. In *X. laevis*, Frizzled 3 is constitutively phosphorylated at Ser576 in a DVL-dependent manner by an unknown kinase. Mutation of C-terminal Ser residues, including Ser576, significantly enhances Frizzled 3 signalling, which suggests that this phosphorylation event downregulates Frizzled signalling[60]. Moreover, Frizzled receptors are regulated by ubiquitylation and deubiquitylation[61], and this modulates cellular responsiveness to WNT (or Wingless in *D. melanogaster*) via the R-spondin WNT agonists[22,23] (see below).

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A remarkable regulation mechanism of Frizzled has been observed during *D. melanogaster* synaptogenesis, a process that requires WNT signalling[62]. In this case, signalling occurs following cleavage of the Frizzled 2 ICD by an unknown ADAM (a disintegrin and metalloprotease) and subsequent nuclear import of Frizzled 2. WNT3 does not regulate the cleavage of Frizzled 2 but instead stimulates nuclear translocation of its ICD. This domain then forms foci containing large ribonucleoprotein (RNP) granules that harbour synaptic protein transcripts. Intriguingly, these Frizzled 2–RNP granules exit the nucleus by budding through the inner and the outer nuclear membranes and export large RNP granules into the cytoplasm[63]. It will be interesting to see whether this mechanism occurs in other cell types and in mammalian cells. Interestingly, a similar transduction pathway has been reported for the WNT receptor RYK during mammalian cortical neuron development (see below).

LRP5 and LRP6 co-receptors. LRP5 and LRP6 and their *D. melanogaster* homologue Arrow are members of the LRP family and act as co-receptors in β-catenin-dependent signalling, in which they are the key regulatory nodes[10]. LRP6 is a large modular, single transmembrane protein and is the best-studied LRP. Its extracellular domain (ECD) mediates the interaction with WNT and Frizzled, leading to ternary complex formation, whereas WNT by itself is not a good LRP6 ligand. The ECD contains multiple, independent WNT-binding sites, which allow different WNTs to bind simultaneously in conjunction with Frizzled[64].

The main mechanism of regulating LRP6 function is through phosphorylation of its ICD, a modification essential for LRP6 function. When overexpressed the ICD is sufficient to activate β-catenin-dependent signalling[64,65]. The ICD contains five Pro-Pro-Pro-Ser/Thr-Pro (PPPSP) repeats, phosphorylation of which is required for β-catenin-dependent signalling[65,66]. Numerous Pro-directed kinases can phosphorylate PPPSP sites and prime LRP6 for further phosphorylation and signalling in response to acute WNT signalling. For example, GSK3, the master negative regulator of β-catenin, mediates phosphorylation of PPPSP sites in response to WNT signalling and thus has both a positive and negative role in the β-catenin-dependent pathway[65]. Similarly, GPCR kinase 5 (GRK5) and GRK6 are membrane-associated PPPSP kinases that are required for WNT signalling[67]. By contrast, other protein kinases phosphorylate PPPSP sites independently of WNT. For example, parathyroid hormone (PTH) binding to its receptor PTH1R induces association of the PTH–PTH1R complex with LRP6 and phosphorylation of PPPSP sites by protein kinase A, which in turn triggers WNT signalling[68]. Mitogen-activated protein kinases (MAPKs) phosphorylate PPPSP sites in response to phorbol esters and fibroblast growth factors (FGFs) to regulate the levels of LRP6 phosphorylation and β-catenin-dependent signalling[69]. Moreover, the cyclin-dependent kinase CDK14 induces phosphorylation of the PPPSP motif in a cell cycle-dependent manner, peaking in G2 phase to M phase of the cell cycle[70]. This is mediated by membrane-tethered G2-M cyclin Y, which recruits CDK14 to LRP6. Therefore, both the competence of LRP6 to respond to incoming WNT signals and canonical WNT signalling itself peak at G2 to M phase.

Phosphorylation of PPPSP repeats by the aforementioned Pro-directed kinases primes LRP6 for phosphorylation by members of the CKI family (notably CKIγ) at adjacent Ser residues (PPPSPXS), and this dual phosphorylation is required for WNT signalling[71]. CKIγ is unique within the CKI family because it is bound to the inner leaflet of the plasma membrane via a C-terminal lipid modification, facilitating LRP6 interaction. CKIγ-mediated phosphorylation of LRP6 is additionally regulated and depends on oligomerized DVL proteins and the formation of LRP signalosomes (FIG. 4), as well as on PtdIns(4,5)P_2[72], transmembrane protein 198 (REF. 73) and APC membrane recruitment protein 1 (AMER1; also known as WTX)[74]. Once LRP6 is fully phosphorylated at PPPSPXS sites, it binds Axin, which recruits the β-catenin destruction complex.

So how does phosphorylated LRP6 inhibit GSK3 to stabilize β-catenin? Activated LRP6 forms signalosomes[57], which inhibit GSK3-mediated phosphorylation of β-catenin in two ways. First, and probably as an immediate response to WNT stimulation, phosphorylated LRP6 binds to and directly inhibits GSK3 (REF. 75). Second, within a few hours GSK3 is sequestered in multivesicular bodies (MVBs), which protect β-catenin from phosphorylation. Unphosphorylated β-catenin escapes ubiquitylation and proteasomal degradation, which allows it to accumulate in the cytoplasm and nucleus[35] (FIG. 4).

WNT binding also induces CKII-mediated phosphorylation of LRP6 on the highly conserved Ser1579 (REF. 76). This allows the endocytic adaptor Disabled 2 (DAB2) to bind LRP6 and negatively regulate the co-receptor by shunting it from caveolin- towards clathrin-dependent endocytosis.

LRP6 may also be regulated through cleavage by complement component C1q[77]. In the presence of C1q, the LRP6 ECD is cleaved, which activates β-catenin-dependent signalling, presumably by generating a constitutively active receptor. However, given the high amounts of C1q that are required for efficient WNT activation *in vitro* (50–100 μg per ml), the physiological relevance of this phenomenon remains unclear.

ROR1 and ROR2. ROR1 and ROR2 are single transmembrane receptor Tyr kinases that contain a CRD similar to that of Frizzled receptors, and the Tyr kinase activity is required for signalling in many but not all circumstances[78,79]. ROR1 and ROR2 bind WNT5A and mostly transduce PCP signalling in vertebrates[37,45], and in *C. elegans* ROR can function cell non-autonomously to sequester WNT[80]. Mice mutated for both *Ror1* and *Ror2* phenocopy *Wnt5a* mutants, indicating that WNT5A is the principal ROR ligand *in vivo*[36,37]. WNT5A binding induces homodimerization of ROR2 (REF. 81) and the formation of a ternary complex with Frizzled[38,39]. This is followed by recruitment of DVL, Axin and GSK3, which activate the receptor[38,46,82]. ROR2 is also activated

Caveolin
A membrane protein that is part of specialized rafts that form flask-shaped, cholesterol-rich invaginations of the plasma membrane. These mediate the uptake of extracellular material and are probably involved in cell signalling.

Clathrin-dependent endocytosis
A pathway that mediates the internalization of plasma membrane proteins. Receptor clustering in membrane domains containing a polymeric clathrin coat and a complex of adaptor proteins and GTPases leads to membrane invagination and scission to form a clathrin-coated vesicle containing the internalized receptors.

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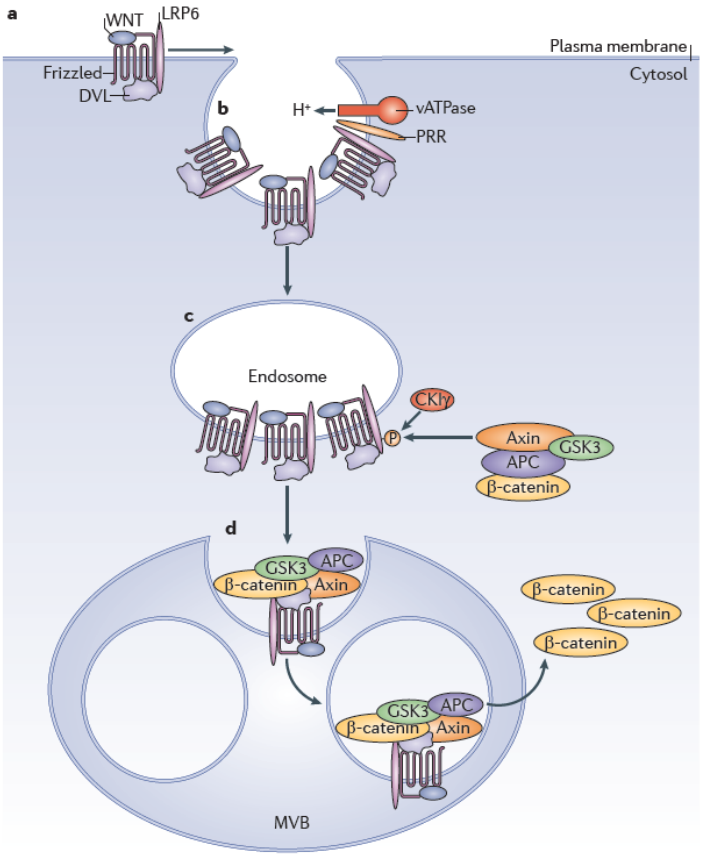

Figure 4 | **LRP6 signalosomes, acidification and MVBs.** **a** | WNT ligands form a ternary complex with Frizzled and low-density lipoprotein receptor-related protein 6 (LRP6), and these complexes become further clustered on platforms of oligomerized Dishevelled (DVL), forming LRP6 signalosomes. **b** | Internalization of the WNT–receptor complex ensues, engaging prorenin receptor (PRR; also known as ATP6AP2), which links WNT receptors to vacuolar ATPases (vATPases). vATPase promotes vesicle acidification, which is essential for vesicle traffic and WNT signalling. **c** | Receptor clustering triggers phosphorylation of the LRP6 intracellular domain (ICD) by casein kinase 1γ (CK1γ), probably by enhanced avidity of the kinase for the receptor supercomplex. The phosphorylated ICD is recognized by Axin, which recruits the destruction complex, including glycogen synthase kinase 3 (GSK3) and adenomatosis polyposis coli (APC). **d** | Endocytosed signalosomes mature into multivesicular bodies (MVBs), requiring the ESCRT (endosomal sorting complex required for transport) complex (not shown). In MVBs the receptor complex containing bound GSK3 is sequestered from the cytoplasm, which protects β-catenin from phosphorylation. Unphosphorylated β-catenin escapes ubiquitylation and proteasomal degradation, which allows it to accumulate in the cytoplasm and nucleus. The figure is modified, with permission, from REF. 131 © (2010) Cell Press.

by forced dimerization[78], as is the case for many receptor Tyr kinases. Downstream, ROR2 recruits the actin-binding protein filamin A and activates JNK, which in fibroblasts leads to cytoskeletal rearrangements and polarized cell migration[83].

Like LRP6, ROR receptors are regulated by GSK3 phosphorylation in a WNT5A-dependent manner[38,84], which activates PCP signalling. ROR2 phosphorylation by TAK1 (TGFβ-activated protein kinase 1) inhibits β-catenin-dependent signalling through phosphorylation at an unknown site (effects on PCP signalling were not assessed in this study)[85]. Moreover, ROR2 autophosphorylates on Tyr outside its Tyr kinase domain. This autophosphorylation event activates its kinase activity, as ROR2 mutants lacking this residue fail to phosphorylate the downstream substrate GRK2 (REF. 86). ROR2 Tyr autophosphorylation was reported to be stimulated by CKIε phosphorylation[86], but in a follow-up experiment CKI inhibitors failed to inhibit ROR2 phosphorylation, unlike GSK3 inhibitors[84].

Although WNT–ROR signalling resembles PCP signalling phenotypically and molecularly, the two pathways may not always overlap. For example, ROR1- and ROR2-mutant cells have normal JNK signalling, and the phenotype of mice lacking both proteins differs from that of mice mutant for the PCP component VANG-like 2 (VANGL2)[87]. Hence, whether PCP and WNT–ROR signalling overlap may depend on the cellular context.

PTK7. PTK7 (also known as Off-track (OTK) in *D. melanogaster*) is another WNT-binding single-pass transmembrane protein[12]. It has an incomplete intracellular kinase domain, which is required for its downstream signalling activities, but seems to be kinase-dead. PTK7 binds Frizzled in the presence of WNTs and can recruit DVL to the plasma membrane[88,89]. PTK7 has been strongly implicated in PCP signalling and, like many PCP signalling activators, is a negative regulator of β-catenin-dependent WNT signalling[12]. However, there is evidence that PTK7 binds to β-catenin and activates β-catenin-dependent signalling in *X. laevis* embryos, thereby affecting neural patterning[90]. The regulation of PTK7 is largely unknown.

RYK. Another single-pass transmembrane receptor Tyr kinase that binds WNTs is RYK (known as Derailed (DRL) in *D. melanogaster*)[91,92], signalling through which has an important role in the central nervous system, notably in axon guidance and neuronal cell fate determination[15,25]. RYK is implicated in β-catenin-dependent, PCP and WNT–Ca^{2+} signalling[91,93,94], and, as is the case with other WNT receptors, RYK signalling can involve DVL[91,95]. RYK is an atypical receptor Tyr kinase and harbours an inactive Tyr kinase domain, and, similarly to PTK7, is kinase-dead. Nevertheless, it can engage SRC Tyr kinase[96], so it is possible that this enzyme promotes phosphorylation of target proteins by recruiting SRC.

Like other WNT receptors, RYK is regulated by post-translational modifications. Specifically, the E3 ubiquitin ligase Mind bomb 1 (MIB1) seems to ubiquitylate RYK constitutively and reduces its plasma membrane levels. This removal of RYK leads to β-catenin activation, presumably because it inhibits PCP signalling and thereby derepresses β-catenin signalling[94].

Moreover, RYK activity is modulated through cleavage of its ICD by γ-secretase[62]. This allows RYK to translocate to the nucleus, where it regulates transcription independently of β-catenin. Similarly to *D. melanogaster* Frizzled 2 (see above), WNT3 does not regulate the cleavage of RYK but instead stimulates the nuclear translocation of its ICD[62].

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MUSK. MUSK acts as a WNT receptor specifically in muscle, where it initiates neuromuscular junction formation as part of a larger receptor complex[14,97]. Like ROR, MUSK has an extracellular region with homology to the Frizzled CRD, binding of which by WNT11 stimulates a PCP-like pathway during neuromuscular development[14,97]. Specifically, MUSK restricts postsynaptic acetylcholine receptors to the centre of the long skeletal muscle cells[14,97]. Similarly, in mammalian muscle cells WNT4, WNT9A and WNT11 regulate acetylcholine receptor clustering through an LRP4-dependent mechanism involving MUSK dimerization and Tyr phosphorylation[98,99]. The biglycan heparan sulphate proteoglycan (HSPG) (see below) is a MUSK ligand, which is required for proper acetylcholine receptor cluster formation[100].

Heparan sulphate proteoglycans. HSPGs have been implicated as co-receptors for various growth factors such as Hedgehog and FGF, but they also bind WNTs[101]. These cell surface macromolecules contain a protein core with sulphated glycosaminoglycan (GAG) chains, which are crucial for WNT binding[102]. Notably, glypicans and syndecans, which are subfamilies of HSPGs, act as WNT co-receptors, as they bind both the ligand and Frizzled and enhance WNT signalling[103–105]. Like other WNT co-receptors, syndecan 4 directly interacts with DVL during PCP signalling[106]. The *D. melanogaster* glypicans Dally and Dally-like promote β-catenin-dependent signalling, but in vertebrates evidence suggests that glypicans are required in PCP signalling[15].

Like other co-receptors, glypicans are regulated through proteolytic processing. The proteins are lipid-anchored in the outer plasma membrane and can be released from the cell surface by the lipase Notum (a potent WNT inhibitor), and this restricts β-catenin-dependent signalling[107,108]. Similarly, enzymes involved in GAG biosynthesis and degradation such as glycosyltransferases, sulphate transporters and an extracellular sulphatase affect WNT signalling[109–112].

Modulation by endocytosis and R-spondins

WNT receptor signalling and endocytosis. In many growth factor signalling pathways, ligand binding triggers endocytosis of the ligand–receptor complex, and it has become clear that this is not only a mode for down-regulation of the signal but also a requirement for signal transduction to occur. For example, TGFβ–SMAD signalling requires endocytosis for presentation of SMAD2 and SMAD3 to the receptor complex by the endosome-specific factor SARA (SMAD anchor for receptor activation)[113]. Likewise, both β-catenin-dependent and β-catenin-independent signalling require endocytosis[15].

In β-catenin-dependent signalling, LRP6 is internalized through a caveolin-mediated route in response to WNT3A, and blocking caveolin-mediated endocytosis inhibits signalling[18,57,114,115,76]. In addition, there is evidence that clathrin-mediated endocytosis of WNT and Frizzled receptors enhances β-catenin-dependent signalling following WNT stimulation[116–118], so it is possible that the cell context determines the endocytic route.

In the case of PCP signalling, findings indicate that only clathrin-mediated endocytosis of WNT–receptor complexes is involved in potentiating the signal. Indeed, interfering with this endocytic pathway inhibits signalling triggered by several receptors, including Frizzled, ROR2, RYK, MUSK, in various cell types[95,97,119,120]. In general, although most studies focus either on endocytosis of a WNT ligand, a Frizzled receptor or a co-receptor, it is likely that entire super-complexes comprising WNT bound to Frizzled and co-receptors are co-internalized.

Consistent with the importance of endocytosis in PCP signalling, the WNT agonist R-spondin 3 promotes clathrin-mediated endocytosis of Frizzled, and this is essential for signalling[18]. Further stressing the close relationship between WNT signalling and endocytosis, endosomal acidification is essential for both β-catenin-dependent and -independent WNT signalling. Remarkably, the WNT receptors Frizzled and LRP6 are directly bound to the acidifying enzyme vacuolar ATPase via the adaptor protein prorenin receptor (PRR; also known as ATP6AP2)[121,122].

Many of these studies support a 'two endocytic routes–two outcomes' model[123], in which caveolin promotes β-catenin-dependent signalling, whereas clathrin-dependent endocytosis promotes PCP signalling. So why is endocytosis required to enhance WNT signalling? In the case of β-catenin-dependent signalling, phosphorylated LRP6 signalosomes that contain Axin and GSK3 are internalized by endocytosis and are then sequestered by intraluminal vesicle budding of late endosomes in MVBs[35] (FIG. 4). This sequestration prolongs the half-life of many GSK3 protein substrates that would otherwise be degraded via the proteasome, including β-catenin. By contrast, for PCP signalling it remains enigmatic why internalization of the WNT–receptor complex is essential for signal transduction to occur.

Endocytosis is not only required for WNT receptor signalling, but it is also a node for regulation of WNT signalling. For example, Frizzled receptors are constitutively removed by the transmembrane RING finger ubiquitin ligases ZNRF3 and RNF43, an event that is counteracted by R-spondins, and this removal downregulates WNT signalling[22]. Similarly, the co-receptor LRP6 is internalized by the transmembrane protein Kremen. The WNT antagonist DKK1 forms a ternary complex with LRP6 and Kremen, which is rapidly endocytosed, thereby removing the WNT co-receptor from the plasma membrane and blocking β-catenin-dependent signalling[124]. Although in many cases DKK1 inhibits WNT signalling in a Kremen-independent manner simply by binding to LRP6, analysis of mice lacking both Kremen 1 and Kremen 2 showed that these proteins are required to inhibit WNT signalling during embryonic limb patterning[125].

WNT receptor regulation by R-spondins. R-spondins were discovered in our laboratory in 2004 as a novel family of secreted WNT agonists[126] (TIMELINE). In an overexpression screen using pools of cDNAs able to

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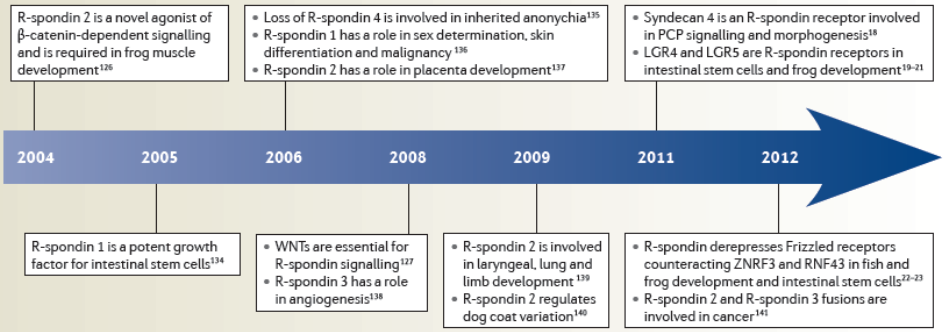
Timeline | **The discovery and characterization of R-spondin**



LGR, Leu-rich repeat-containing G protein-coupled receptor; PCP, planar cell polarity; RNF43, RING finger 43; ZNRF3, zinc and RING finger 3.

activate a WNT responsive reporter plasmid, we initially isolated R-spondin 2, which stimulates β-catenin-dependent signalling and potently enhances the response of cells to low doses of WNT. R-spondins have been shown not only to promote β-catenin-dependent signalling but also PCP signalling[18]. R-spondin 1–R-spondin 4 all have a similar structure and harbour two furin domains, which are necessary and sufficient for β-catenin-dependent signalling[126], and one thrombospondin type I domain, which promotes PCP signalling[18]. Not only do R-spondins synergize with WNTs but in fact require WNTs to elicit downstream effects, as knockdown of Evenness interrupted (EVI; also known as WNTless), which is required for secretion of WNT proteins, abolishes the ability of R-spondin 1 to induce the β-catenin response[127].

R-spondins are developmental regulators but may also act as growth factors for intestinal stem cells and as potential oncogenes[17]. How R-spondins act mechanistically has been debated. They were proposed to bind directly to LRP6 (REF. 128), but this claim could not be confirmed[18,129]. Furthermore, it has been suggested that the DKK1 co-receptor Kremen also acts as a R-spondin receptor[129], but neither could binding be confirmed[18] nor did Kremen double mutant cells display a reduced R-spondin response[125].

However, in 2011 three classes of R-spondin receptors were discovered, and this has provided important insights into the R-spondin mechanism of action. Specifically, syndecans were shown to act as high-affinity receptors for R-spondin 2 and R-spondin 3, as they bind to their thrombospondin domains[18]. In *X. laevis* embryos, syndecan 4 and R-spondin 3 mediate PCP signalling in two morphogenetic processes that involve convergent extension, gastrulation and cartilage extension. How do syndecans activate R-spondin signalling? As discussed above, WNT signalling requires endocytosis, and R-spondin signalling is no exception, as R-spondin–receptor complexes are also internalized via clathrin-mediated endocytosis[18,130]. A hallmark of syndecans is their ability to induce endocytosis following ligand binding. Consistent with this, syndecans trigger R-spondin internalization, probably by clustering WNT receptor complexes and DVL, and this event is essential for R-spondin 3 to induce PCP signalling[18]. By contrast, syndecans do not seem to be involved in β-catenin-dependent signalling, possibly because they show specificity for WNT–Frizzled complexes engaged in PCP signalling.

A second class of R-spondin receptors comprises LGR4, LGR5 and LGR6 (REFS 19–21). LGRs, which until recently were classified as orphan receptors, are exquisite markers of self-renewing stem cells in rapidly proliferating epithelia, such as the intestine and the skin, and this process depends on WNT signalling[17]. Indeed, self-renewal and WNT-responsive gene expression are abolished in intestinal stem cells in mice lacking both LGR5 and LGR6 (REF. 20), strongly suggesting that R-spondins promote stem cell self-renewal via LGRs. Unlike syndecans, LGRs bind the furin domains of R-spondins with high affinity and promote β-catenin-dependent signalling[19,20] and PCP signalling[21]. LGR loss-of-function inhibits β-catenin-dependent signalling following R-spondin but not WNT stimulation. R-spondin–LGR signalling also involves clathrin-mediated endocytosis[21,130], and in the case of hepatic HepG2 cells this is essential for β-catenin-dependent signalling[21].

A clue for how R-spondins and their receptors may enhance WNT signalling came from the discovery of ZNRF3 and RNF43. These proteins were very recently identified as negative feedback regulators of WNT signalling, and they are regulated by R-spondins[22,23]. *ZNRF3* and *RNF43* expression is induced by WNT signalling, and the proteins act as steady state inhibitors of both β-catenin-dependent and PCP signalling[22,23]. Frizzled receptors are regulated by cycles of ubiquitylation and deubiquitylation[61] (see above), and ZNRF3 and RNF43 act as Frizzled ubiquitin ligases, removing Frizzled and possibly LRP6 from the plasma membrane. This is counteracted by R-spondin 1, which induces ZNRF3 internalization[22].

Furin domains
Protein domains of unknown function that are found in the protease furin and in non-proteases.

Thrombospondin type I domain
A protein domain that is found in thrombospondin 1 and in non-matrix proteins. This domain binds to heparan sulphate proteoglycans.

Convergent extension
A process during gastrulation in which layers of cells converge and extend by a rearrangement of the cells of the ventral part of the epithelium towards the ventral midline.

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Whether R-spondins also counteract RNF43 remains to be shown. Overexpression of a dominant-negative ZNRF3 induces defects in anteroposterior patterning in zebrafish and dorsoventral patterning in *X. laevis*. However, this dominant-negative effect may not be ZNRF3-specific, as *Znrf3*-knockout mice show mostly normal development with only lens defects[22]. This is in contrast to the much more dramatic effects observed in early frog development[22]. Conditional double knockout of *ZNRF3* and *RNF43* induces intestinal adenomas[23], indicating that they may compensate for each other.

Taken together, the recent results argue for a model (FIG. 5) in which R-spondins derepress WNT signalling by protecting Frizzled receptors from internalization and degradation. This mode of action would explain why R-spondins cannot signal without low doses of WNT, why endocytosis is required for R-spondin signalling and why R-spondins promote both β-catenin-dependent and PCP signalling.

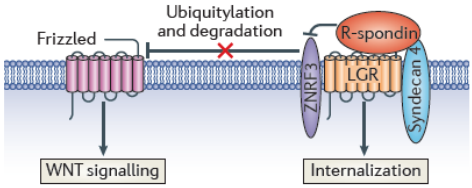

Figure 5 | **Model for R-spondin–WNT signalling.** Zinc and RING finger 3 (ZNRF3) ubiquitylates the WNT receptor Frizzled, targeting a fraction of it for degradation. When R-spondin is present, it binds to its receptors Leu-rich repeat-containing G protein-coupled receptor 4 (LGR4), LGR5 or LGR6 via its furin domains. This results in complex formation with ZNRF3, which is removed from the cell surface, allowing greater accumulation of Frizzled receptor on the plasma membrane and enhancement of WNT signalling. In addition to LGRs, syndecan 4 binding to the thrombospondin I domain of R-spondin is required for enhancement of planar cell polarity (PCP) signalling.

Conclusions and open questions

The question of how the interaction of 19 WNTs with their more than 15 receptors and co-receptors in various permutations triggers a cellular response is at the heart of mechanistic understanding of signalling mediated by this important growth factor family. The elucidation of the three-dimensional structure of WNT bound to the Frizzled CRD has paved the way for a new dimension of biochemical and structural understanding of WNT–receptor interaction. However, it remains unclear which conformational changes in Frizzled receptors are induced across the Frizzled transmembrane domain by WNT binding to transduce the signal intracellularly. Future work should also address the molecular structure of the ternary complexes that are formed between WNT–Frizzled and the various co-receptors, which should be feasibly using their extracellular domains. This may yield important insights that will assist in the design of interfering small molecules of high specificity.

Frizzled proteins seem to act as common receptors for both the β-catenin-dependent and β-catenin-independent pathways, and LRP5 and LRP6 or ROR1 and ROR2 determine β-catenin-dependent and β-catenin-independent pathways, respectively. However, whether ROR1 and ROR2 are always involved in PCP signalling or may be substituted in a cell type-specific manner by PTK7 or RYK remains to be elucidated. Indeed, the number of different molecular responses to the various WNT–co-receptor combinations is staggering, and we need to clarify whether these correspond to wholly different signalling branches or are just manifestations of a small number of core pathways. In other words, efforts should centre on unifying rather than dispersing downstream signalling events. In this respect, one common key component, which interacts with all WNT receptors tested, is DVL.

The model of the LRP6 signalosomes maturing into MVBs and sequestering GSK3 and the surprising role of WNT–Frizzled in the formation and budding of RNP particles highlight the importance of vesicular trafficking in WNT–receptor responses. Hence, the cell biology aspects of WNT–receptor signalling are likely to take centre stage in the future. Correspondingly, WNT signalling proceeds via endosomal compartments, but a rationale for this remains unclear, notably in PCP signalling.

Although recent studies have led to a model for R-spondin-mediated derepression of WNT signalling, many questions remain. It has been suggested that R-spondin 1 acts by tethering ZNRF3 to LGR4 (REF. 22), but it remains to be biochemically confirmed whether R-spondin 1 binds directly to ZNRF3. It is possible that ZNRF3 recognizes R-spondins only in the context of the ligand bound to an LGR or syndecan. Moreover, it seems unlikely that LGR proteins act only by antagonizing ZNRF3 and potentially RNF43, and it is possible that they may have additional signalling functions.

Endocytosis has a key role in the activity of R-spondins. However, how can their ability to induce Frizzled endocytosis via syndecan and LGR4 (REFS 18,21,130) be reconciled with the model that their function is to stabilize Frizzled receptors on the plasma membrane[22]? A dominant negative ZNRF3 inhibits β-catenin-dependent signalling in dorsal axis determination and anteroposterior patterning in *X. laevis* and/or zebrafish embryos. However, these processes are unaffected by R-spondin signalling, despite the presence of LGRs, suggesting that there remain additional cofactors to be discovered.

Finally, because WNT signalling is implicated in various diseases, most importantly cancer, efforts are being made to identify small molecules that interfere with the pathway, ideally in a cell type-specific manner. Kinases that regulate LRP6 phosphorylation are interesting candidates, notably the less promiscuous CKIγ and CDK14. Likewise, the solved structure of WNT in complex with the Frizzled CRD may allow the design of specific WNT antagonists.

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Acknowledgements
The author thanks C. Janda and C. Garcia for providing the WNT–Frizzled CRD structure and apologizes to those authors whose work could not be cited owing to space limitations.

Competing interests statement
The author declares no competing financial interests.

FURTHER INFORMATION
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