

Expression Pattern of Wnt Signaling Components in the Adult Intestine

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Background & Aims: In the intestine, the canonical Wnt signaling cascade plays a crucial role in driving the proliferation of epithelial cells. Furthermore, aberrant activation of Wnt signaling is strongly associated with the development of colorectal cancer. Despite this evidence, little is known about the precise identity and localization of Wnts and their downstream effectors in the adult intestine. To address this issue, we examined the expression pattern of all Wnts, Frizzleds (Fzs), low-density lipoprotein receptor-related proteins, Wnt antagonists, and T-cell factors in the murine small intestine and colon and adenomas. **Methods:** Embryonic, postnatal, and adult intestinal samples were subjected to in situ hybridization by using specific RNA probes for the various genes tested. **Results:** Our analysis showed high expression of several signaling components (including *Wnt-3*, *Wnt-6*, *Wnt-9b*, *Frizzled 4*, *Frizzled 6*, *Frizzled 7*, low-density lipoprotein receptor-related protein 5, and secreted Frizzled-related protein 5) in crypt epithelial cells. We also detected *Wnt-2b*, *Wnt-4*, *Wnt-5a*, *Wnt-5b*, *Frizzled 4*, and *Frizzled 6* in differentiated epithelial and mesenchymal cells of the small intestine and colon. Finally, several factors (*Frizzled 4*, T-cell factor 1, lymphoid enhancer factor, Dickkopf 2, Dickkopf 3, and Wnt-interacting factor) displayed differential expression in normal vs neoplastic tissue. **Conclusions:** Our study predicts a much broader role for Wnt signaling in gut development and homeostasis than was previously anticipated from available genetic studies and identifies novel factors likely involved in promoting canonical and noncanonical Wnt signals in the intestine.

The intestine harbors a variety of cell types that are organized into highly regular structures known as villi and crypts of Lieberkühn. The absorptive and protective functions of the gut are ensured by enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. These differentiated epithelial cells arise from transit-amplifying progenitors and, ultimately, stem cells, both of which reside in the crypts. The intestine also consists of a layer of loose connective tissue, or mesenchyme, which is thought to provide structural support and

growth signals to the overlying epithelium. Finally, embedded within the various layers of the intestine, one additionally finds blood vessels, smooth muscle, and neuronal cells, as well as lymphocytes.

Cellular proliferation and differentiation in the intestine depends on a large array of signaling molecules.^{1,2} One such example includes the Wnt family of secreted growth factors.^{3–5} Wnts are evolutionarily conserved, cysteine-rich glycoproteins capable of signaling in both a paracrine and autocrine fashion. Wnts induce a wide range of biological effects by binding to the 7-span transmembrane protein Frizzled (Fz)⁶ and the single-span low-density lipoprotein receptor-related protein (LRP).^{7–9} In a complex with Fz/LRP, Wnts trigger the release of β -catenin from a so-called destruction complex, which in the absence of Wnts promotes ubiquitin-mediated degradation of β -catenin.³ Subsequently, free β -catenin shuttles to the nucleus, where it associates with DNA-binding factors of the T-cell factor (TCF) family to activate the transcription of target genes.^{10,11} More recent evidence has shown that Wnts may also stimulate cellular responses independently of β -catenin and TCF.^{12,13} Examples of these so-called noncanonical pathways involve either the intracellular release of calcium ions and activation of Ca^{2+} -dependent kinases or morphogenic changes dependent on RhoA and Jun kinase stimulation, also termed the *planar cell polarity pathway*.

Genetic studies have shown an essential role for canonical Wnt signaling in regulating intestinal epithelial cell proliferation. One of the earliest indications of this came from the analysis of mutations in core Wnt signaling components such as adenomatous polyposis coli

Abbreviations used in this paper: APC, adenomatous polyposis coli; Dkk, Dickkopf; ISH, in situ hybridization; Fz, Frizzled; LEF, lymphoid enhancer factor; LRP, low-density lipoprotein receptor-related protein; sFRP, secreted Frizzled-related protein; SSC, standard saline citrate; TCF, T-cell factor; WIF, Wnt-interacting factor.

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(APC) and β -catenin.^{14–16} These genetic alterations lead to the formation of intestinal adenomas as a result of deregulated nuclear accumulation of β -catenin and constitutive activation of target genes associated with proliferation of epithelial cells.^{17–20} Conversely, blockage of canonical Wnt signals in the intestine, either through deletion of *TCF-4* or overexpression of the Wnt antagonist Dickkopf (*Dkk-1*), results in arrested epithelial cell proliferation.^{21–23} Another function ascribed to Wnt signaling in epithelial cells was recently uncovered through DNA microarray analysis. *EphB2* and *B3* are *TCF-4*–responsive genes that regulate upward movement of epithelial cells and the positioning of Paneth cells within the crypt–villus axis.²⁴ Together these data provide clear evidence that proliferation and sorting of crypt epithelial cells depend on the correct dosage of canonical Wnt signals.

Despite the functional evidence described previously, relatively little is known about the localization of Wnt signaling components within the intestine. Expression studies in mice and chicks have shown that during embryonic development, Wnt gene products are broadly expressed throughout the intestinal tract.^{25–27} In the adult intestine, Wnt expression is maintained,²⁸ but their precise localization remains unclear. To address this issue in detail, we screened the expression patterns of all known Wnts, Fz/LRP and Wnt antagonists, and TCF family members in the adult intestine by in situ hybridization (ISH).

Materials and Methods

Probes

The following probes used in this study were described elsewhere: *Wnt-1*^{24,29}; *Wnt-2*, *Wnt-4*, *Wnt-5b*, *Wnt-6*, *Wnt-7a*, and *Wnt-7b*³⁰; *Wnt-3* and *Wnt-3a*³¹; *Wnt-8a*³²; *Wnt-8b*^{32,33}; *Wnt-9a/14* and *Wnt-9b/14b*^{33,34}; *Wnt-10a* and *Wnt-10b*³⁵; *Wnt-11*³⁶; *Fz-4* and *Fz-7*³⁷; *Dkk-1*³⁸; and lymphoid enhancer factor (*Lef*)³⁹. Other probes were derived from reverse-transcription polymerase chain reaction products and correspond to the following nucleotides: 781–1640 for *Wnt-16*, 1488–1815 for *Fz-1*, 1245–1784 for *Fz-2*, 1371–1733 for *Fz-3*, 1500–2500 for *Fz-5*, 418–940 for *Fz-8*, 1296–1864 for *Fz-9*, and 994–1413 for *Fz-10*. The remaining probes correspond to expressed sequence tags obtained from the IMAGE consortium (MRC geneservice, Babraham, United Kingdom). The GenBank accession numbers for these probes are as follows: *Wnt-5a*, 4317623; *Fz-6*, 4973661; secreted Frizzled-related protein 1 (*sFRP-1*), BG975485; *sFRP-2*, BQ958218; *sFRP-3*, AA399912; *sFRP-4*, BC034853; *sFRP-5*, BC032921; *Dkk-2*, BE310466; *Dkk-3*, BQ933260; *Dkk-4*, BC018400; Wnt-interacting factor (*WIF*), BG246236; cerberus, AA120122; *TCF-4*, BG921346; *TCF-1*, BF141520; and *TCF-3*, AA015280. To ensure the specificity of the probes, we gener-

ated both sense and antisense probes for *Wnt-3*, *Wnt-9b*, *Wnt-4*, *Wnt-5b*, *Wnt-6*, *Fz-4*, *Fz-6*, *sFRP-1*, *sFRP-5*, *WIF*, *Dkk-2*, *Dkk-3*, *TCF-4*, *TCF-1*, and *TCF-3* and tested these in parallel in our ISH protocol. Representative examples are shown in Supplementary Figure 1 (see supplementary material online at <http://www.niob.knaw.nl/researchpages/clevers/files/Figure%201%20supplementary.jpg>; see supplementary Figure legend online at <http://www.niob.knaw.nl/researchpages/clevers/files/Figure%20Legends%20Supplementary%20Figure%201%20and%202.pdf>).

In Situ Hybridization

Intestines from normal or *APC^{min}* mice (C57BL/6, 3 months old) were flushed and fixed overnight in formalin. Samples were then dehydrated and embedded in paraffin, sectioned at 8 micrometers, and processed for hybridization as described below. Sections were dewaxed, rehydrated, treated with 0.2N HCl, digested in proteinase K solution, postfixed, treated in acetic anhydride solution, and hybridized overnight for 24–48 hours at 68°C with various probes in 5× standard saline citrate (SSC; pH 4.5), 50% formamide, 2% blocking powder (Roche, Almere, The Netherlands), 5 mmol/L ethylenediaminetetraacetic acid, 50 μ g/mL yeast transfer RNA, 0.1% Tween 20, 0.05% 3[3-cholaminopropyl diethylammonio]-1-propane sulfonate, and 50 μ g/mL heparin. Sections were then rinsed in 2× SSC and washed for 3 × 20 minutes at either 60°C or 65°C in 2× SSC/50% formamide. After several rinses in tris-buffered saline/Tween, sections were then blocked for 30 minutes in tris-buffered saline/Tween containing 0.5% blocking powder (Roche). Next, sections were incubated in blocking solution overnight at 4°C with alkaline phosphatase–conjugated anti-digoxigenin (1:2000 dilution; Roche). After washing several times in tris-buffered saline/Tween, the color reaction was performed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution. For image analysis, sections were temporally mounted in glycerol or permanently mounted after dehydration in Pertex (Klinipath, The Netherlands). A complete protocol will be provided upon request. In vitro transcription reactions to generate labeled probe was performed as follows: 1 μ g of linearized DNA was incubated at 37°C for more than 2 hours with 4 μ L of transcription buffer (Promega, Leiden, The Netherlands), 2 μ L of dithiothreitol 0.1 mol/L (Promega), 2 μ L of Dig RNA labeling mix (Roche), 1 μ L of ribonuclease inhibitor (Promega), and 1.5 μ L of T7, T3, SP6 (Promega) in a total volume of 20 μ L.

Antibody Staining

The immunohistochemistry procedure is described elsewhere.²⁴ Briefly, sections were pretreated with peroxidase blocking buffer (120 mmol/L Na₂HPO₄, 43 mmol/L citric acid, 30 mmol/L NaN₃, and 0.2% H₂O₂; pH 5.8) for 20 minutes at room temperature. Antigen retrieval was performed by boiling samples in sodium citrate buffer (10 mmol/L; pH 6.0). After 20 minutes, the boiling pan was allowed to slowly

cool down to room temperature. Incubation of antibodies was performed in 1% bovine serum albumin in phosphate-buffered saline overnight at 4°C. The primary antibodies used in this study were rabbit anti-lysozyme (1:500; Dako, Glostrup, Denmark) and goat anti-TCF-4 (1:500; Santa Cruz, Santa Cruz, CA). Rabbit anti-goat (Dako) and rabbit EnVision+ (Dako) were used as secondary antibodies.

Results

Expression of Wnt Genes in the Adult Intestine

To provide a comprehensive expression profile of Wnt signaling components in the intestinal mucosa, we first collected and generated RNA probes for all murine Wnts (19 genes), Fzs (10 genes), LRP5 (2 genes), TCFs (4 genes), sFRPs (5 genes), Dkks (4 genes), WIF, and cerberus (see Materials and Methods). By ISH on sections, we initially examined the expression of Wnt genes in both embryonic and adult stages. Of the 19 Wnt probes tested, 7 Wnts were readily detected in the intestine (results are recapitulated in Figure 1 and Supplementary Table 1, see supplementary material online at <http://www.niob.knaw.nl/researchpages/clevers/files/Supplementary%20Table%201.pdf>). As shown in Figures 2 and 3, Wnt ligands are expressed in both the epithelial and mesenchymal layers of the intestine. In the epithelium, *Wnt-3* was associated with the very bottom of the crypts of Lieberkühn, at the sites where Paneth cells are located (Figure 2A). Similarly, in the distal portion of the small intestine, *Wnt-9b* (also termed *Wnt-14b*) was found predominately at the bottom of the crypts (Figure 2B). To confirm the identity of these cells, we performed immunostainings on consecutive 4-μm sections for the classic Paneth cell marker lysozyme. As shown on corresponding sections (Figure 2C and D) of the same intestinal crypt, *Wnt-9b*-expressing cells also produced lysozyme and thus represent Paneth cells. It is interesting to note that in more proximal areas of the small intestine, *Wnt-9b* was also detected in epithelial progenitor cells above the Paneth cell compartment (Figure 2E). Contrary to the small intestine, in the colon, *Wnt-9b* was localized throughout the colonic epithelium (Figure 2F). Moreover, both *Wnt-3* and *9b* displayed weak expression in adenomas of *APC^{min}* mice (data not shown). Note that a recent report has additionally observed *Wnt-9b* expression in the fetal gut.⁴⁰ Finally, we found *Wnt-6* expressed throughout the crypts of the small intestine and colon (Figure 2G and H). Similarly, *Wnt-6* was strongly expressed in adenomas (Figure 2I).

Several Wnts were also found in specific compartments of the mesenchyme. In the fetal gut, *Wnt-5a*

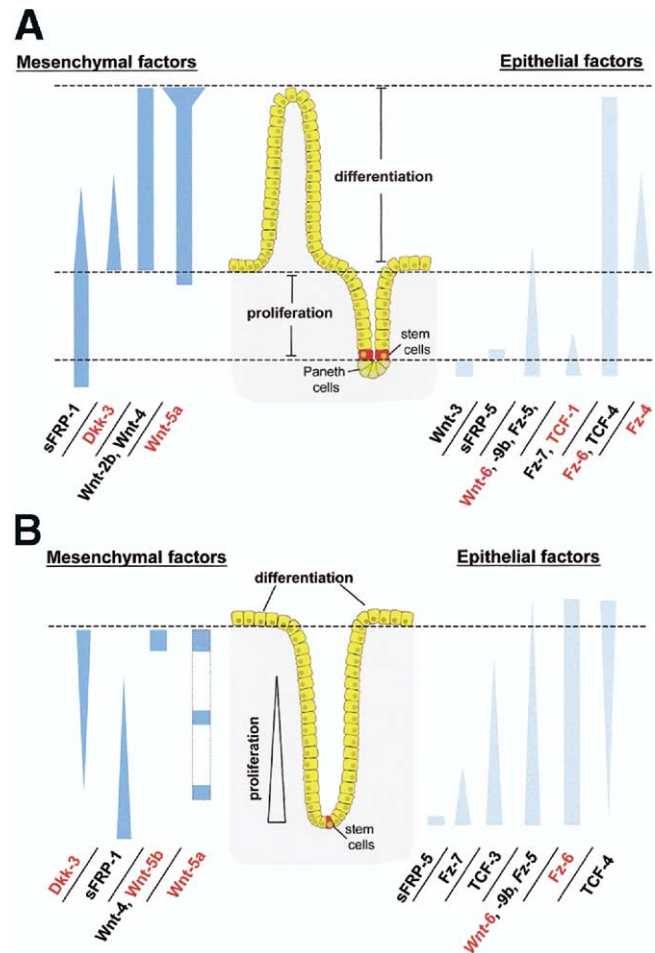


Figure 1. Summary of expression data. (A) and (B) show schematic representations of the small intestine and colon, respectively. The epithelium is shown in yellow, and the mesenchymal layer is depicted in gray. Epithelial compartments associated with proliferation and differentiation are also shown. For simplicity, only the expression patterns of Wnt signaling components that are most relevant to epithelial proliferation/differentiation are shown. Factors highlighted in red are also up-regulated in adenomas. Other factors such as *WIF*, *Dkk-2*, and *LEF* are not expressed in the normal intestine but are ectopically expressed in adenomas.

showed high expression at the tips of growing villi and in few discrete cells beneath the proliferative epithelium (Figure 3A). In the adult small intestine, *Wnt-5a* was similarly abundant in the villus tips, although weaker expression was also observed throughout the villi, as well as at the crypt–villus junction (Figure 3B). In the colon, *Wnt-5a* and the closely related *Wnt-5b* were restricted to the mesenchyme beneath the surface epithelium (Figure 3C and F). Finally, *Wnt-5a* and, to a lesser extent, *Wnt-5b* were both up-regulated in stromal cells of *APC^{min}* polyps (Figure 3D and E and data not shown). Unlike *Wnt-5a*, *Wnt-4* was uniformly expressed along the villus mesenchyme and was absent from adenomas (Figure 3G and data not shown). However, in the

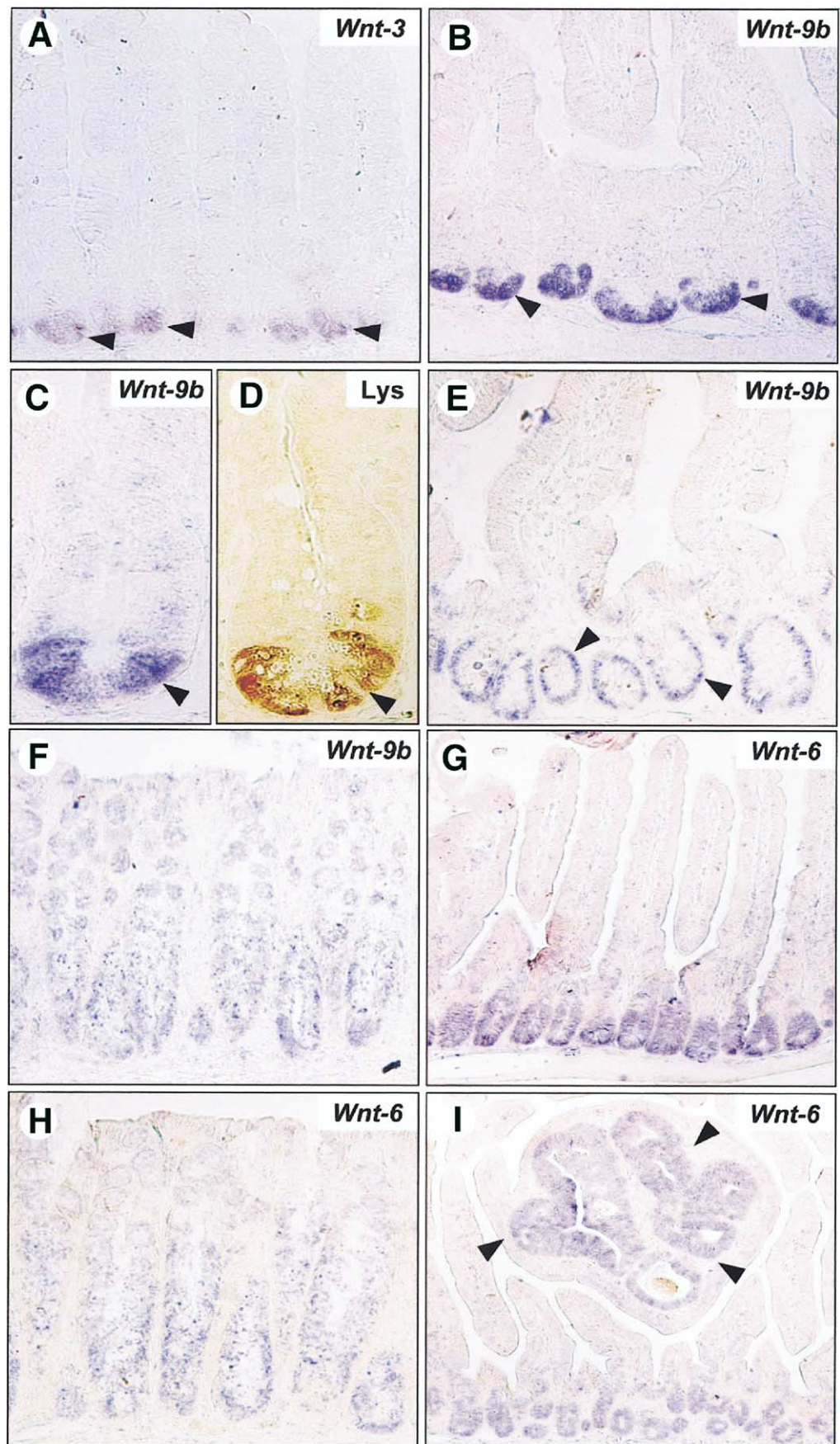


Figure 2. ISH analysis of Wnts expressed in intestinal epithelial cells. (A) shows *Wnt-3* expressed in Paneth cells (arrowheads). (B and C) show *Wnt-9b* strongly and predominately expressed in Paneth cells in the distal portion of the intestine (ileum). (D) represents a consecutive 4- μ m section of the same intestinal crypt as shown in (C) that was immunostained with an antibody recognizing the Paneth cell marker lysozyme. (E) represents a section of the duodenum and shows *Wnt-9b* expression in Paneth cells and crypt progenitor cells. (F) shows *Wnt-9b* expressed throughout the colonic epithelium. (G, H, and I) show *Wnt-6* in crypt epithelial cells of the small intestine, colon, and adenomas, respectively.

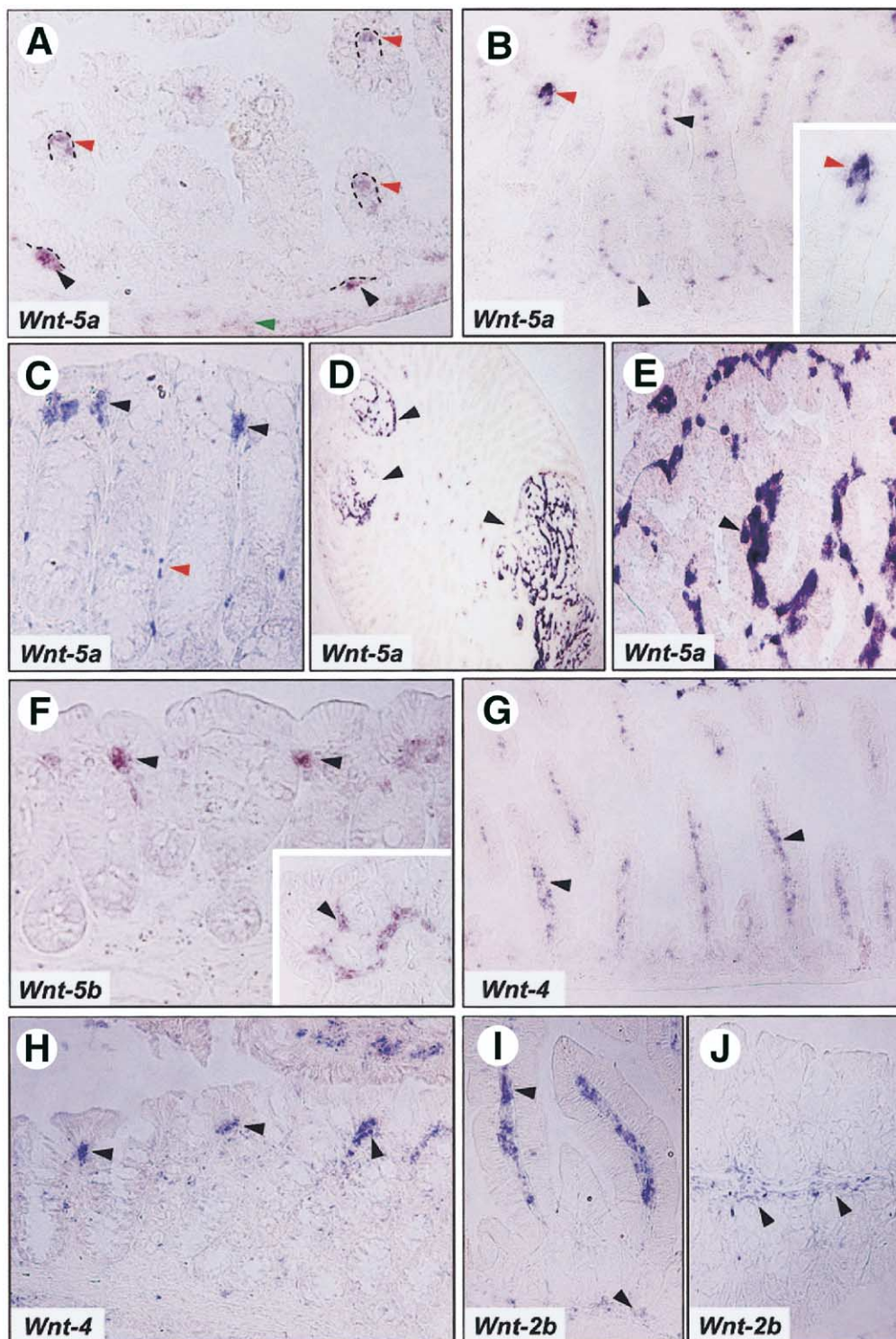


Figure 3. ISH analysis of Wnts expressed in the mesenchyme of the small intestine and large intestine. (A) shows at E18.5 *Wnt-5a* accumulation in the mesenchyme of the villi tips (red arrowheads), in mesenchymal cells adjacent to proliferative epithelial cells (black arrowheads), and in the smooth muscle layer (green arrowhead). The border between the epithelium and the mesenchyme is indicated with dashed lines. In the mature intestine (B), *Wnt-5a* is strongly expressed in mesenchymal cells at the tips of villi (red arrowheads and inset), whereas levels are reduced along the villi toward the crypt-villus border (black arrowheads). (D) and a higher magnification in (E) show *Wnt-5a* up-regulation in stromal cells of intestinal polyps (black arrowheads). (C, F, and H) show *Wnt-5a*, *Wnt-5b*, and *Wnt-4*, respectively, just beneath the surface epithelium of the colon. Note that *Wnt-5a* is also detected lower in the crypts (red arrowheads). Inset in (F) shows a cross section of colonic crypts with *Wnt-5b* staining in the surrounding mesenchyme. (G) shows uniform *Wnt-4* expression throughout the villi. (I and J) show *Wnt-2b* in mesenchymal cells of the small intestine and endothelial or smooth muscle cells of the colon, respectively.

colon, *Wnt-4* expression closely resembled *Wnt-5a* and *5b* stainings (Figure 3H). Finally, *Wnt-2b* was strongly expressed in the mesenchymal layer of the villi and more weakly in the crypts of the small intestine (Figure 3I). In the colon, *Wnt-2b* transcripts were less abundant and seemed to be markers of endothelial or smooth muscle cells (Figure 3J).

Expression of Wnt Receptors in the Adult Intestine

To identify putative Wnt-responsive cells, we next examined the expression of Fz-related receptors 1–10. As indicated in Supplementary Table 1 (see supplementary material online at <http://www.niob.knaw.nl/>

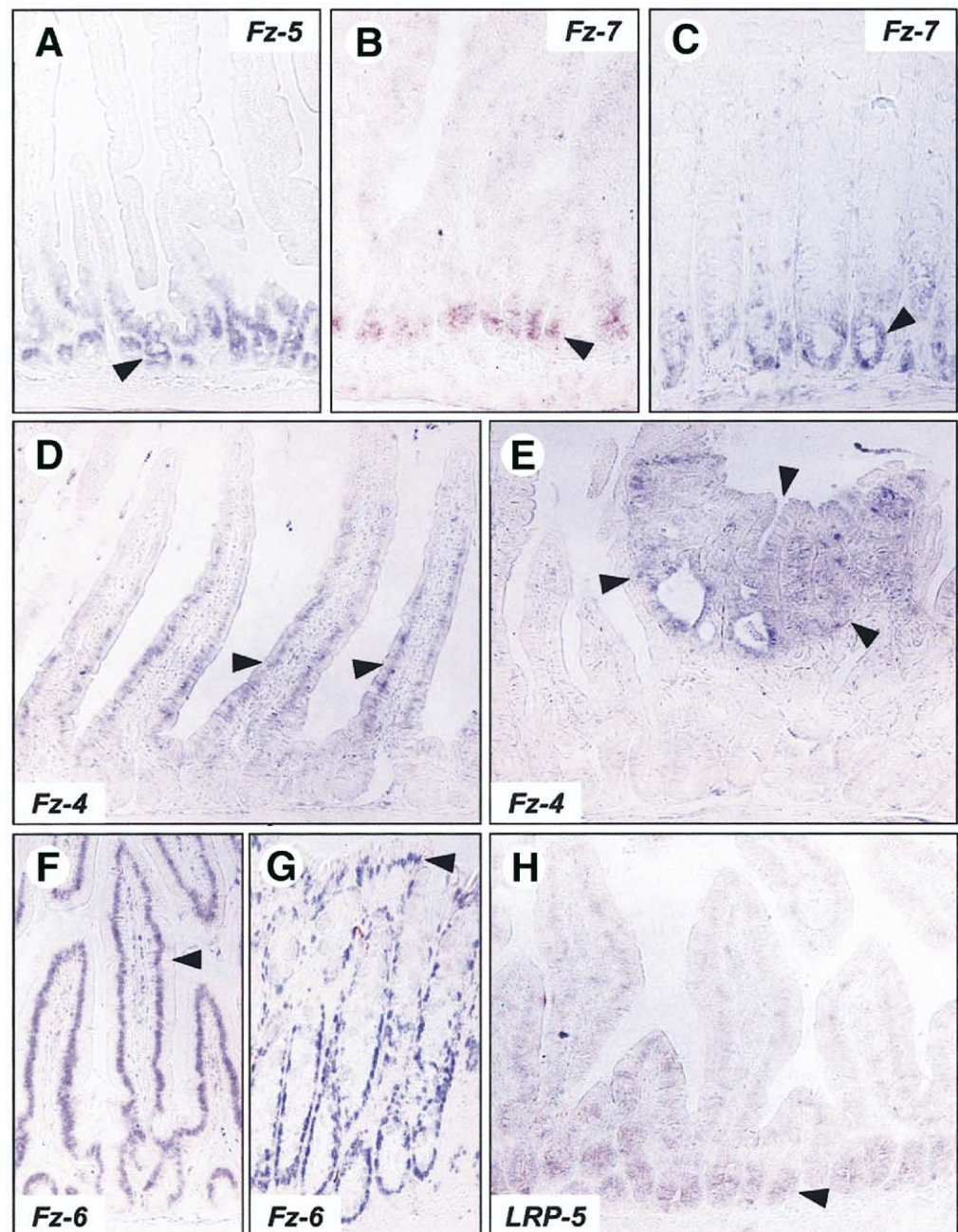


Figure 4. ISH analysis of Wnt receptors in the intestine and colon. (A) *Fz-5* in the epithelium of the crypts and crypt-villus border. (B and C) *Fz-7* in the lower portions of adult intestinal and colonic crypts, respectively. (D) *Fz-4* in differentiated epithelial cells of the villi. Note that expression of *Fz-4* is strongest in the lower half of the villi. (E) *Fz-4* up-regulated in intestinal polyps. (F and G) *Fz-6* throughout the epithelium in the small and large intestine, respectively. (H) *LRP-5* restricted to proliferative epithelial cells.

researchpages/clevers/files/Supplementary%20Table%201.pdf) and Figure 4, several *Fz* genes were located in both the fetal and adult intestine. As shown in Figure 4A and in van Es et al,⁴¹ *Fz-5* was detected in epithelial cells of the intervillus pockets of fetal guts and in the crypts of adult intestines. In addition, *Fz-7* and *Fz-4* were dynamically expressed during intestinal development. *Fz-7* was strongly expressed in the smooth muscle layer and in intervillus epithelial cells of neonates (P1; data not shown), although at later stages, *Fz-7* was confined to the epithelium of the crypt bottom (Figure 4B and C). *Fz-4* transcripts were also widely distributed in neonatal guts

(Supplementary Table 1, see supplementary material online at <http://www.niob.knaw.nl/researchpages/clevers/files/Supplementary%20Table%201.pdf>), whereas expression in adults was comparatively weaker and restricted to differentiated epithelial cells of the villi (Figure 4D). One notable exception to this was the high levels of *Fz-4* in adenomas, as shown in Figure 4E. Finally, *Fz-6* was uniformly expressed throughout the epithelium of the small and large intestine (Figure 4F and G). We also tested the expression of the LRP coreceptors *LRP-5* and *LRP-6*, given their absolute requirement in driving canonical

Wnt signals. As expected, we found both *LRP-5* and *LRP-6* expressed in proliferative epithelial cells of the crypts (Figure 4H and data not shown).

Expression of Secreted Wnt Antagonists in the Adult Intestine

The functional counterparts of the Fz receptors include a family of secreted factors termed sFRPs.^{42,43} Both Fzs and sFRPs share an equivalent Wnt-interacting, cysteine-rich domain, which allows sFRPs to compete with Fzs and thereby antagonize Wnt signaling. Of the 5 sFRP probes tested here, we detected expression of only sFRP-1 and sFRP-5. In the small intestine and colon, mesenchymal cells immediately adjacent to the crypts, as well as cells within the submucosa, expressed abundant levels of sFRP-1 (Figure 5A and B and data not shown). sFRP-5 was strongly expressed in epithelial progenitor cells at E16.5 (Figure 5C). However, in the mature intestine, not all proliferative cells expressed sFRP-5. Indeed, sFRP-5 transcripts were detected in single cells in positions within the intestinal and colonic crypts reminiscent of stem cells^{44–46} (Figure 5D and E). In some instances, we also found weaker sFRP-5 expression throughout the crypt bottom of the small intestine (data not shown). To further define the localization of sFRP-5-expressing cells within the crypts, double stainings were performed for both sFRP-5 and lysozyme (Supplementary Figure 2C, see supplementary material online at <http://www.niob.knaw.nl/researchpages/clevers/files/Figure%20%20supplementary.jpg>; see supplementary Figure legend online at <http://www.niob.knaw.nl/researchpages/clevers/files/Figure%20Legends%20Supplementary%20Figure%201%20and%202.pdf>). This approach showed that cells expressing sFRP-5 were located immediately above the Paneth cell compartment. We were also interested in comparing the expression of sFRP-5 with that of the proposed intestinal stem cell marker musashi 1.^{47–49} To test this, we examined both sFRP-5 and musashi 1 expression by ISH on consecutive sections. As shown in Supplementary Figure 2A and B, (see supplementary material online at <http://www.niob.knaw.nl/researchpages/clevers/files/Figure%20%20supplementary.jpg>; see supplementary Figure legend online at <http://www.niob.knaw.nl/researchpages/clevers/files/Figure%20Legends%20Supplementary%20Figure%201%20and%202.pdf>) we found that sFRP-5 displayed a more localized expression pattern than musashi 1. Indeed, the latter was expressed throughout the epithelial progenitor cell compartment. Finally, it is worth noting that sFRP-1 and sFRP-5 were not detected in intestinal polyps (data not shown).

Like sFRPs, other secreted factors can inhibit Wnt action by binding directly to Wnts, as in the case of WIF and cerberus,^{42,43} or by binding to LRP coreceptors, as with the Dkk class.⁵⁰ In Figure 5F, we found WIF uniquely expressed in adenomas, thus confirming a recent report.⁵¹ Similarly, a *Dkk-2* probe specifically stained adenomas (Figure 5G), whereas *Dkk-3* was weakly expressed in the villus mesenchyme and myenteric plexuses and up-regulated in adenomas (Figure 5H and I). *Dkk-1*, *Dkk-4*, and cerberus were undetected in the intestinal tract (data not shown).

Expression of T-Cell Factor Family Members in the Intestine

Numerous studies have examined the function of TCFs in gut development and cancer.⁵² As shown in Figure 6A and B, a *TCF-4* probe showed staining along the entire crypt–villus axis, as well as in the enteric nervous system. *TCF-4* expression was also maintained in adenomas (data not shown). In the colon, *TCF-4* transcripts were most abundant in differentiated cells of the surface epithelium, whereas expression diminished in the lower half of the crypts. Because a role for TCF-4 in nonproliferative cells was not anticipated on the basis of the available genetic data,²¹ we wished to confirm the ISH results with TCF-4 antibody stainings. When comparing equivalent regions within the colon (Figure 6C and D), we once again found TCF-4 expressed predominantly in noncycling cells away from the crypt bottom.

Another TCF family member previously implicated in regulating epithelial proliferation is TCF-1. Indeed, *APC^{min}* mice crossed into a *TCF-1*-null background display increased numbers of intestinal polyps. This implies that TCF-1 acts downstream of TCF-4/ β -catenin and may play a role in carcinogenesis.⁵³ In agreement with this, we found that *TCF-1* expression was strongly up-regulated in adenomas when compared with normal proliferative crypt cells (Figure 6G). However, we also observed *TCF-1* transcripts in infiltrating gut lymphocytes, gut-associated lymphoid tissue, and Peyer patches (Figure 6E and F). This result is supported by previous findings showing a crucial role for TCF-1 in thymocyte development.⁵⁴ As with *TCF-1*, we also detected *LEF* expression in intestinal polyps, although in normal epithelium, *LEF* transcripts were absent (Figure 6H). Similar findings were observed in human colorectal tumors, in which *LEF* expression is directly regulated by TCF-4/ β -catenin.⁵⁵ Finally, we also examined expression of *TCF-3*. Northern blot analysis has shown that *TCF-3* is mainly expressed in the caudal portion of the intestinal tract.⁵⁶ Likewise, our analysis showed expression of *TCF-3* in the proliferative compartment of the colon,

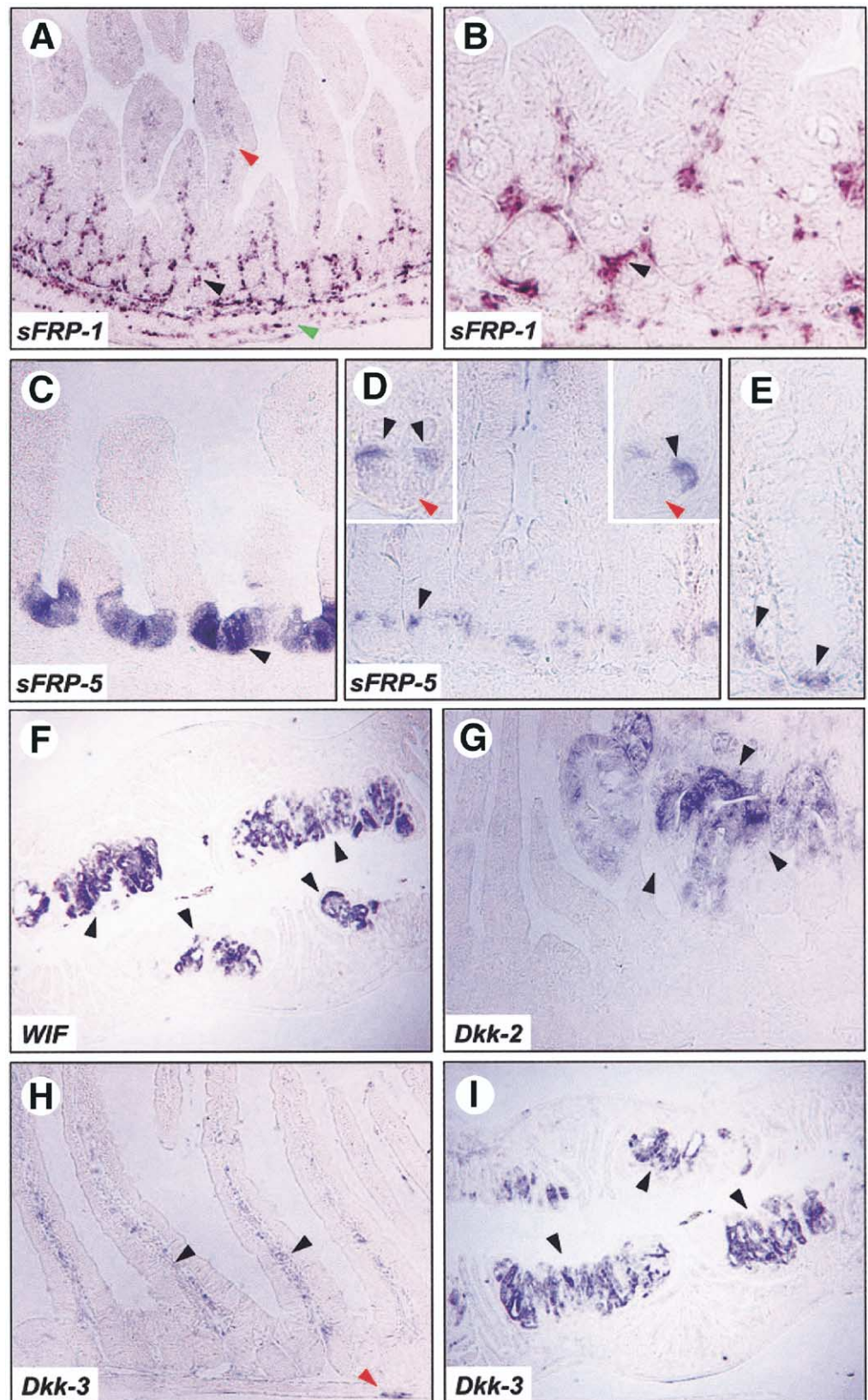


Figure 5. ISH analysis of secreted Wnt antagonists in the small intestine. (A) and the close-up in (B) show *sFRP-1* in mesenchymal cells surrounding the crypts. Additional spotty staining is also detected in the smooth muscle layer (green arrowhead), whereas expression in the villi is reduced (red arrowhead). (C) shows *sFRP-5* expression in all proliferative epithelial cells of the fetal (E16.5) intestine. (D) shows *sFRP-5* staining of single crypt epithelial cells in the adult small intestine. Insets show close-ups of 2 typical examples of *sFRP-5* expression in single cells (black arrowheads) just above the Paneth cells (red arrowheads). (E) shows *sFRP-5* expression limited to the base of 2 consecutive colonic crypts. (F and G) show *WIF* and *Dkk-2*, respectively, expressed only in intestinal polyps of *APC^{min}* mice (black arrowheads), whereas normal epithelium is devoid of any expression. (H) shows *Dkk-3* expressed in mesenchymal cells of the villi (black arrowhead) and plexuses of the enteric nervous system (red arrowhead). (I) shows *Dkk-3* strongly up-regulated in *APC^{min}* polyps.

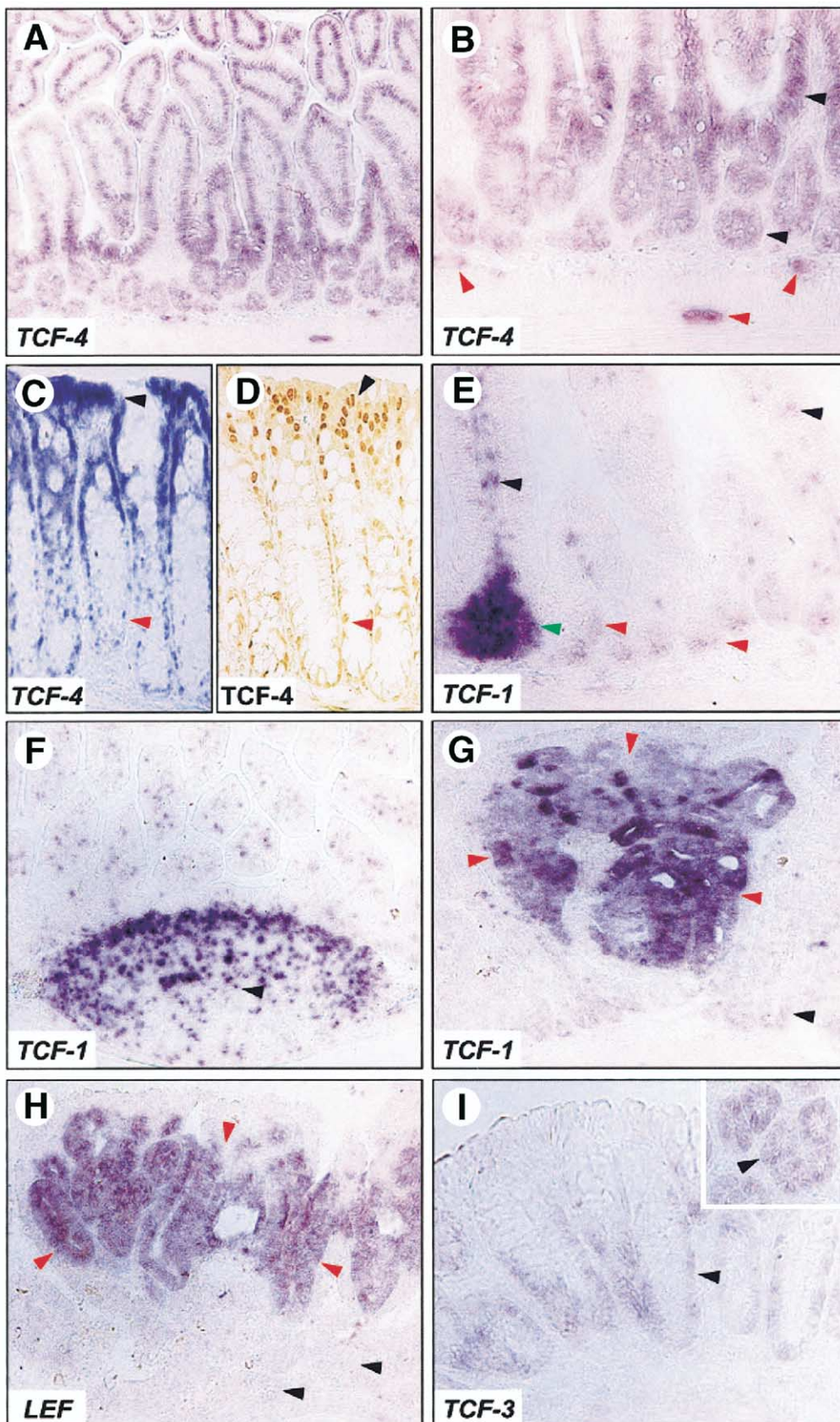


Figure 6. ISH analysis of TCFs in the small and large intestine. (A) and the higher magnification in (B) show *TCF-4* throughout the epithelium of the small intestine (black arrowheads). *TCF-4* also accumulates in the submucosal and myenteric ganglia of the enteric nervous system (red arrowheads). (C) shows high expression of *TCF-4* in the upper half or noncycling areas of the colonic crypts (black arrowheads), whereas expression is reduced in the lower half the crypts (red arrowheads). (D) shows a similar expression gradient with *TCF-4* antibodies. (E) shows *TCF-1* in epithelial cells of the crypt bottom (red arrowheads), in gut-associated lymphoid tissue (GALT; green arrowhead), and in putative infiltrating intestinal lymphocytes (black arrowheads). (F) shows high expression of *TCF-1* in Peyer patches. (G) shows up-regulation of *TCF-1* in adenomas (red arrowheads) when compared with normal crypt epithelium (black arrowheads). (H) shows ectopic expression of *LEF* in intestinal polyps (red arrowheads), whereas normal epithelium is devoid of *LEF* (black arrowheads). (I) and inset show unique *TCF-3* expression in epithelial cells of colonic crypts.

although we were unable to detect *TCF-3* elsewhere (Figure 6I).

Discussion

In this article, we present an exhaustive overview of the expression pattern of Wnt signaling components in the murine intestine. As summarized in Figure 1 and Supplementary Table 1 (see supplementary material online at <http://www.niob.knaw.nl/researchpages/clevers/files/Supplementary%20Table%201.pdf>), Wnts and their downstream effectors are confined to specific mesenchymal and epithelial compartments of the intestine. Given these observations, the function of at least some of these factors may be inferred.

Canonical and Noncanonical Wnt Signaling in the Gut

In Figure 2 we have shown that in the small intestine, *Wnt-3* and *Wnt-9b* are expressed in Paneth cells. In Paneth cells, nuclear β -catenin is readily detected and is thought to activate expression of the repulsion/guidance receptor EphB3.²⁴ EphB3, in combination with its ligand ephrin B1, forces Paneth cells to occupy the very bottom of the crypts. Therefore, on the basis of this evidence, *Wnt-3* and *Wnt-9b* may act in an autocrine loop to activate TCF/ β -catenin target genes involved in positioning of Paneth cells within the crypt-villus axis. *Wnt-3* and *Wnt-9b* may also be important in promoting the maturation of Paneth cells. We and others have recently shown that expression of Paneth cell markers, such as antimicrobial peptides (ie, cryptdins and defensins), is dependent on active Wnt/ β -catenin signals.^{41,57}

Alternatively, *Wnt-3* and *Wnt-9b*, as well as *Wnt-6* expressed in crypt cells, may signal to epithelial progenitor cells lying above Paneth cells. In turn, these canonical Wnts would drive proliferation, possibly through receptors identified here, such as Fz-5, Fz-7, and LRPs (Figure 4). Along the same lines, recent immunohistological studies and in vitro functional assays have identified *Wnt-11* as another candidate implicated in promoting proliferation.⁵⁸

Contrary to the Wnts discussed previously, our study has also identified candidate Wnts likely involved in inducing noncanonical signals, such as *Wnt-2b*, *Wnt-4*, *Wnt-5a*, and *Wnt-5b*. These Wnts are expressed in the villus mesenchyme in the small intestine and/or the mesenchyme adjacent to the surface epithelium in the colon (Figure 3). In these regions, epithelial and mesenchymal cells are devoid of nuclear β -catenin, and TCF target genes are turned off, thus

implying that these Wnts do not activate β -catenin/Tcf in the intestine.⁵⁹ Several lines of evidence suggest that noncanonical Wnt signals regulate a complex array of cellular responses, including increased calcium flux, repression of TCF-mediated transcription, and cytoskeletal rearrangements.^{12,13} Therefore, in the intestine, *Wnt-2b*, *Wnt-4*, *Wnt-5a*, and *Wnt-5b* could conceivably inhibit proliferation and promote differentiation of epithelial cells, induce cellular polarity, and promote villus formation. The expression pattern of *Wnt-5a* in the gut combined with recent functional analysis illustrates this point particularly well. Indeed, experiments in transfected cells and *Wnt-5a*-deficient limb buds have shown that *Wnt-5a* promotes degradation of β -catenin and suppresses TCF-activated genes.⁶⁰ In the fetal gut and adenomas, *Wnt-5a* is expressed in mesenchymal cells closely associated with highly proliferative epithelial cells. Thus, in this context, *Wnt-5a* may function to down-regulate β -catenin and in turn attenuate proliferation.

The actions of noncanonical Wnts discussed previously may be mediated by Fz-4 and Fz-6. Indeed, both receptors are expressed in the differentiated epithelial cells of the villi, in close proximity to *Wnt-2b*-, *Wnt-4*-, *Wnt-5a*-, and *Wnt-5b*-producing cells (Figure 4). Similarly, in adenomas, Fz-4 and *Wnt-5a* are up-regulated in epithelial and stromal cells, respectively. Considering that Fz-4 is known to physically interact with *Wnt-5a*,⁶¹ these factors may coordinate cross talk between epithelial and mesenchymal cells. Finally, functional and biochemical evidence suggests that Fz-6 is an important mediator of planar cell polarity signals in mammalian epithelial cells⁶² and that it can actively repress canonical Wnt signals.⁶³

Factors Modulating Wnt Signaling in the Intestine

Wnt signaling in self-renewing tissues such as the intestine is likely to be tightly regulated. Our screen of secreted Wnt antagonists has identified several factors that may fulfill this function. *sFRP-1*, for example, was localized to the crypt mesenchyme, coexpressed and adjacent to cells abundantly expressing Wnts (Figure 5). Consequently, *sFRP-1* may function to modulate the effects of Wnt signaling in these regions. Indeed, a recent survey showed that *sFRP-1* was frequently down-regulated in colorectal cancers; this implies a tumor-suppressor role for this factor.⁶⁴ Moreover, forced expression of *sFRPs* blocks proliferation of colorectal cell lines.⁶⁵ Similarly, WIF, as well as *Dkk-2* and *Dkk-3*, may also play a role in tumorigenesis, on the basis of their ectopic expression in adenomas (Figure 5). However, how these

factors may be implicated in adenoma formation is unclear, given that Wnt signaling is constitutively turned on in these cells. Further complicating the matter, in vivo data have shown that Dkk-3 is unable to block Wnt signaling,^{66,67} whereas Dkk-2 can synergize with Fz receptors to induce Wnt signaling responses.^{68,69} Finally, *sFRP-5* was strongly expressed in single epithelial cells of the bottom of crypts (Figure 5 and Supplementary Figure 2). Labeling experiments have shown that stem cells, the number of which may vary from 1 to 6 per crypt, are located in equivalent positions just above the Paneth cells in the small intestine and at the crypt base in the colon.^{44–46} We are presently investigating whether *sFRP-5*-positive cells represent bona fide stem cells by gene deletion in the mouse germ line.

Diverse Functions for T-Cell Factors in the Gut

In this study, we have identified several previously unknown sites within the intestinal mucosa harboring TCF family members. TCF-1, for example, is proposed to function as a tumor suppressor that regulates the proliferation of epithelial cells in the intestine.⁵³ This is confirmed by our ISH data, which also point to a possible role for TCF-1 in gut lymphocytes. Similarly, the expression of *TCF-4* in cellular compartments other than epithelial progenitors, such as the enteric nervous system and mature enterocytes (Figure 6), suggests a broader role for TCF-4 in the gut. This notion is particularly evident in the colon, where *TCF-4* was found mainly in differentiated cell types. In several systems, TCF transcription factors are known to both activate and repress transcription.⁷⁰ Accordingly, TCF-4 in differentiated β -catenin-inactive cells may be required to suppress target genes associated with proliferation. However, Tcf-4 is also likely to play a role in maintaining proliferation of colonocytes. This assertion is based on the observation that in *Tcf-4*^{-/-} embryos, proliferative epithelial cells are absent in the proximal portion of the colon (data not shown). The detection of *TCF-3* in the colon in an inverse gradient to that of *TCF-4* also poses an interesting question. Given the genetic data showing that TCF-3 primarily functions as a transcriptional repressor,⁷¹ it will be interesting to ascertain the role of TCF-3 in relation to TCF-4 in the colon.

In conclusion, our comprehensive overview of Wnt signaling in the intestine has identified for the first time specific Wnts and downstream signaling components likely involved in controlling both canonical and noncanonical pathways. In turn, this study shows a broad role for Wnt signaling in regulating multiple

aspects of intestinal development, homeostasis, and carcinogenesis.

References

- de Santa BP, van den Brink GR, Roberts DJ. Development and differentiation of the intestinal epithelium. *Cell Mol Life Sci* 2003; 60:1322–1332.
- Sancho E, Batlle E, Clevers H. Signaling pathways in intestinal development and cancer. *Annu Rev Cell Dev Biol* 2004;20:695–723.
- Logan CY, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004;20:781–810.
- Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. *Genes Dev* 1997;11:3286–3305.
- Wodarz A, Nusse R. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* 1998;14:59–88.
- Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. *Nature* 1996;382:225–230.
- Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 2000;407:535–538.
- Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. LDL-receptor-related proteins in Wnt signal transduction. *Nature* 2000;407:530–535.
- Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, Vaizel-Ohayon D, Schejter E, Tomlinson A, DiNardo S. Arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* 2000;407:527–530.
- Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 1996;382:638–642.
- Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, Roose J, Destree O, Clevers H. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* 1996;86:391–399.
- Strutt D. Frizzled signalling and cell polarisation in *Drosophila* and vertebrates. *Development* 2003;130:4501–4513.
- Veeman MT, Axelrod JD, Moon RT. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* 2003;5:367–377.
- Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* 2003;1653:1–24.
- Kolligs FT, Bommer G, Goke B. Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion* 2002;66:131–144.
- Tejpar S, Cassiman JJ, Van Cutsem E. The molecular basis of colorectal cancer. *Acta Gastroenterol Belg* 2001;64:249–254.
- Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* 2004;18:1385–1390.
- van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002;111:241–250.
- Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 1997;275:1784–1787.
- Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon

- cancer by mutations in beta-catenin or APC. *Science* 1997;275:1787–1790.
21. Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf4. *Nat Genet* 1998;19:379–383.
 22. Kuhnert F, Davis CR, Wang HT, Chu P, Lee M, Yuan J, Nusse R, Kuo CJ. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A* 2004;101:266–271.
 23. Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* 2003;17:1709–1713.
 24. Battle E, Henderson JT, Begthel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 2002;111:251–263.
 25. Lickert H, Kispert A, Kutsch S, Kemler R. Expression patterns of Wnt genes in mouse gut development. *Mech Dev* 2001;105:181–184.
 26. McBride HJ, Fatke B, Fraser SE. Wnt signaling components in the chicken intestinal tract. *Dev Biol* 2003;256:18–33.
 27. Theodosiou NA, Tabin CJ. Wnt signaling during development of the gastrointestinal tract. *Dev Biol* 2003;259:258–271.
 28. Holcombe RF, Marsh JL, Waterman ML, Lin F, Milovanovic T, Truong T. Expression of Wnt ligands and Frizzled receptors in colonic mucosa and in colon carcinoma. *Mol Pathol* 2002;55:220–226.
 29. McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 1990;62:1073–1085.
 30. Gavin BJ, McMahon JA, McMahon AP. Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev* 1990;4:2319–2332.
 31. Roelink H, Nusse R. Expression of two members of the Wnt family during mouse development—restricted temporal and spatial patterns in the developing neural tube. *Genes Dev* 1991;5:381–388.
 32. Bouillet P, Oulad-Abdelghani M, Ward SJ, Bronner S, Chambon P, Dolle P. A new mouse member of the Wnt gene family, mWnt-8, is expressed during early embryogenesis and is ectopically induced by retinoic acid. *Mech Dev* 1996;58:141–152.
 33. Richardson M, Redmond D, Watson CJ, Mason JO. Mouse Wnt8B is expressed in the developing forebrain and maps to chromosome 19. *Mamm Genome* 1999;10:923–925.
 34. Katoh M. Molecular cloning and expression of mouse Wnt14, and structural comparison between mouse Wnt14-Wnt3a gene cluster and human WNT14-WNT3A gene cluster. *Int J Mol Med* 2002;9:221–227.
 35. Wang J, Shackleford GM. Murine Wnt10a and Wnt10b: cloning and expression in developing limbs, face and skin of embryos and in adults. *Oncogene* 1996;13:1537–1544.
 36. Kispert A, Vainio S, Shen L, Rowitch DH, McMahon AP. Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. *Development* 1996;122:3627–3637.
 37. Wang Y, Macke JP, Abella BS, Andreasson K, Worley P, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene frizzled. *J Biol Chem* 1996;271:4468–4476.
 38. Monaghan AP, Kioschis P, Wu W, Zuniga A, Bock D, Poustka A, Delius H, Niehrs C. Dickkopf genes are co-ordinately expressed in mesodermal lineages. *Mech Dev* 1999;87:45–56.
 39. Oosterwegel M, van de Wetering M, Timmerman J, Kruisbeek A, Destree O, Meijlink F, Clevers H. Differential expression of the HMG box factors TCF-1 and LEF-1 during murine embryogenesis. *Development* 1993;118:439–448.
 40. Qian J, Jiang Z, Li M, Heaphy P, Liu YH, Shackleford GM. Mouse Wnt9b transforming activity, tissue-specific expression, and evolution. *Genomics* 2003;81:34–46.
 41. van Es JH, Jay P, Gregorieff A, van Gijn ME, Jonkheer S, Hatzis P, Thiele A, van den Born M, Begthel H, Brabletz T, Taketo MM, Clevers H. Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat Cell Biol* 2005;7:381–386.
 42. Jones SE, Jomary C. Secreted Frizzled-related proteins: searching for relationships and patterns. *Bioessays* 2002;24:811–820.
 43. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003;116:2627–2634.
 44. Bach SP, Renahan AG, Potten CS. Stem cells: the intestinal stem cell as a paradigm. *Carcinogenesis* 2000;21:469–476.
 45. Marshman E, Booth C, Potten CS. The intestinal epithelial stem cell. *Bioessays* 2002;24:91–98.
 46. Potten CS. The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. *Cancer Metastasis Rev* 1992;11:179–195.
 47. Nishimura S, Wakabayashi N, Toyoda K, Kashima K, Mitsufuji S. Expression of Musashi-1 in human normal colon crypt cells: a possible stem cell marker of human colon epithelium. *Dig Dis Sci* 2003;48:1523–1529.
 48. Kayahara T, Sawada M, Takaishi S, Fukui H, Seno H, Fukuzawa H, Suzuki K, Hiai H, Kageyama R, Okano H, Chiba T. Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine. *FEBS Lett* 2003;535:131–135.
 49. Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, Ashton G, Clarke R, Sakakibara S, Okano H. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. *Differentiation* 2003;71:28–41.
 50. Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol* 2001;3:683–686.
 51. Cebrat M, Strzadala L, Kisielow P. Wnt inhibitory factor-1: a candidate for a new player in tumorigenesis of intestinal epithelial cells. *Cancer Lett* 2004;206:107–113.
 52. Waterman ML. Lymphoid enhancer factor/T cell factor expression in colorectal cancer. *Cancer Metastasis Rev* 2004;23:41–52.
 53. Roose J, Huls G, van Beest M, Moerer P, van der Horn K, Goldschmeding R, Logtenberg T, Clevers H. Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* 1999;285:1923–1926.
 54. Verbeek S, Izon D, Hofhuis F, Robanus-Maandag E, te Riele H, van de Wetering M, Oosterwegel M, Wilson A, MacDonald HR, Clevers H. An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 1995;374:70–74.
 55. Hovanes K, Li TW, Munguia JE, Truong T, Milovanovic T, Lawrence MJ, Holcombe RF, Waterman ML. Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. *Nat Genet* 2001;28:53–57.
 56. Korinek V, Barker N, Willert K, Molenaar M, Roose J, Wagenaar G, Markman M, Lamers W, Destree O, Clevers H. Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol Cell Biol* 1998;18:1248–1256.
 57. Andreu P, Colnot S, Godard C, Gad S, Chafey P, Niwa-Kawakita M, Laurent-Puig P, Kahn A, Robine S, Perret C, Romagnolo B. Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development* 2005;132:1443–1451.
 58. Ouko L, Ziegler TR, Gu LH, Eisenberg LM, Yang VW. Wnt11 signaling promotes proliferation, transformation, and migration

- of IEC6 intestinal epithelial cells. *J Biol Chem* 2004;279:26707–26715.
59. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002;111:241–250.
 60. Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. *J Cell Biol* 2003;162:899–908.
 61. Chen W, ten Berge D, Brown J, Ahn S, Hu LA, Miller WE, Caron MG, Barak LS, Nusse R, Lefkowitz RJ. Dishevelled 2 recruits beta-arrestin 2 to mediate Wnt5A-stimulated endocytosis of Frizzled 4. *Science* 2003;301:1391–1394.
 62. Guo N, Hawkins C, Nathans J. Frizzled6 controls hair patterning in mice. *Proc Natl Acad Sci U S A* 2004;101:9277–9281.
 63. Golan T, Yaniv A, Bafico A, Liu G, Gazit A. The human Frizzled 6 (HFz6) acts as a negative regulator of the canonical Wnt. beta-catenin signaling cascade. *J Biol Chem* 2004;279:14879–14888.
 64. Caldwell GM, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, Chughtai S, Wallis Y, Matthews GM, Morton DG. The Wnt antagonist sFRP1 in colorectal tumorigenesis. *Cancer Res* 2004;64:883–888.
 65. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Dong CW, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004;36:417–422.
 66. Krupnik VE, Sharp JD, Jiang C, Robison K, Chickering TW, Amaravadi L, Brown DE, Guyot D, Mays G, Leiby K, Chang B, Duong T, Goodearl AD, Gearing DP, Sokol SY, McCarthy SA. Functional and structural diversity of the human Dickkopf gene family. *Gene* 1999;238:301–313.
 67. Mao B, Niehrs C. Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene* 2003;302:179–183.
 68. Brott BK, Sokol SY. Regulation of Wnt/LRP signaling by distinct domains of Dickkopf proteins. *Mol Cell Biol* 2002;22:6100–6110.
 69. Wu W, Glinka A, Delius H, Niehrs C. Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling. *Curr Biol* 2000;10:1611–1614.
 70. Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 1998;395:608–612.
 71. Merrill BJ, Pasolli HA, Polak L, Rendl M, Garcia-Garcia MJ, Anderson KV, Fuchs E. Tcf3: a transcriptional regulator of axis induction in the early embryo. *Development* 2004;131:263–274.

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