WNT SIGNALING IN GUT DEVELOPMENT AND HOMEOSTASIS

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Wnt signaling in gut development and homeostasis

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Deep Thoughts…

"...man will occasionally stumble over the truth, but usually manages to pick himself up, walk over or around it, and carry on." *Winston Churchill*

"Science is a wonderful thing if one does not have to earn one's living at it."
Albert Einstein

"A mouse is an animal that, if killed in sufficiently many and creative ways, will generate a PhD." *Anonymous*

For my parents

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CHAPTER 1: INTRODUCTION

Wnt signaling in the intestinal epithelium:

from endoderm to cancer

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Wnt signaling in the intestinal epithelium: from endoderm to cancer

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Abstract

The Wnt pathway controls cell fate during embryonic development. It also persists as a key regulator of homeostasis in adult self-renewing tissues. In these tissues, mutational deregulation of the Wnt cascade is closely associated with malignant transformation. The intestinal epithelium represents the best-understood example for the closely linked roles of Wnt signaling in homeostatic self-renewal and malignant transformation. In this review, we outline current understanding of the physiological role of Wnt signaling in intestinal biology. From this perspective, we then describe how mutational subversion of the Wnt cascade leads to colorectal cancer (CRC).

Introduction

Development and homeostasis in all multi-cellular organisms depend on a complex interplay between processes involved in cell proliferation, migration, differentiation, adhesion, and death. This diverse array of cellular responses is in large part coordinated by a relatively small number of intercellular signals, examples of which include the BMP, TGF, Notch, Hh, and Wnt pathways. One of the major developments in recent years has been the realization that the signaling pathways triggered by these factors are very often deregulated in pathological conditions (1-6). This notion is particularly well illustrated by the role of the Wnt pathway in the intestinal epithelium. The relevance of Wnt signaling to intestinal biology was established, unknowingly at the time, over ten years ago when the tumor suppressor gene Adenomatous polyposis coli (APC) was found mutated in a large number of hereditary and sporadic cases of CRC (7-9). Subsequently, combined work from several laboratories led to the finding that inactivation of APC in CRC cells results in constitutively active Wnt signaling (10-12). Since these early findings a much richer picture has emerged. It is now recognized that Wnt signaling not only drives tumorigenesis but is also required at different stages of gut development, as well as during adult epithelial homeostasis. Our approach in this review will be to dissect the different functions attributed to Wnt signaling at these various time points. First, we shall begin by introducing some of the components of the pathway most relevant to our discussion.

A short summary of the Wnt pathway

Wnts and their downstream effectors were originally discovered in *Drosophila* and subsequently shown to be conserved in all metazoans (13). Genetic and biochemical data taken from these models has, to date, identified over 50 proteins directly involved in transducing Wnt signals (see Wnt homepage at www.stanford.edu/~rnusse/ wntwindow.html). How these proteins interact with one another to stimulate various biological responses has been an area of intense investigation.

Wnt genes, of which there are 19 in man and mice, encode for cysteine-rich glycoproteins. Production of biologically active Wnts depends on palmitoylation of a conserved cysteine residue (14). This process may be mediated by Porcupine/MOM1, however direct proof for this has not yet been provided (15-17). Once released into the extracellular milieu, Wnts interact with secreted proteins such as SFRPs and WIF (18). In general, these factors are thought to function as inhibitors by sequestering Wnts and preventing their interaction with membrane-bound receptors. Other interaction partners include membrane-anchored heparan sulfate proteoglycans (HSPGs). In *Drosophila*, the HSPG Dally acts as a positive regulator of Wnt activity, but its precise biochemical function is unknown (19).

Wnts activate responding cells by interacting with the seven-span transmembrane protein Frizzled (Fz) and the single-span transmembrane protein LRP (20-23). Two functional complexes involving these proteins have been described. Wnts may simultaneously

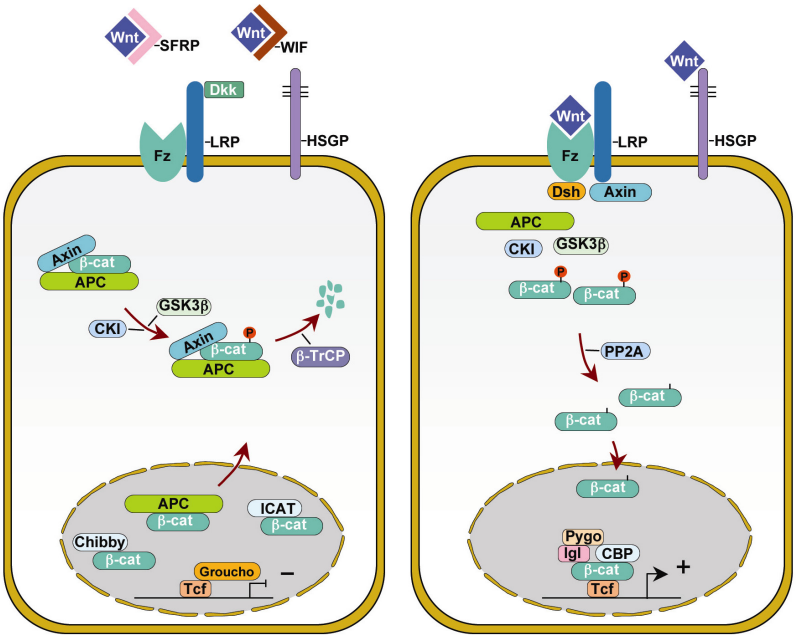

Figure 1. The Wnt Canonical Pathway. Left: in the absence of Wnt stimulation β-catenin levels are kept at a minimum through the destruction complex composed of APC, Axin, GSK3β, and CKI. In the nucleus Tcf factors associate with transcriptional repressors to block target gene activation. Right: in the presence of Wnt stimulation, the destruction complex is destabilized and β-catenin accumulates in the nucleus to activate transcription of Tcf target genes.

bind to Fz and LRP. This represents the initial step in the so-called canonical pathway, which leads to the formation of nuclear Tcf/β-catenin complexes. Alternatively, when LRP is not expressed or downmodulated through secreted factors such as Dickkopfs (24), Wnts may nonetheless form a complex with Fz, triggering Tcf/β-catenin-independent cellular responses such as increased calcium flux, repression of Tcf-mediated transcription and cytoskeletal rearrangements. Collectively, these responses are often referred to as non-canonical signaling (25). As of yet, this aspect of Wnt signaling has not been analyzed in the gut. For this reason, non-canonical Wnt signaling will not be covered in this review.

The key component of the Wnt canonical cascade is the cytoplasmic protein β-catenin. In the absence of Wnts, the scaffolding proteins APC and Axin/Axin2 sequester β-catenin allowing casein kinase I (CKI) to phosphorylate the N-terminus of β-catenin at serine S45, a residue often mutated in cancers (26;27). Subsequently, glycogen synthase kinase 3 beta (GSK3β) is recruited to phosphorylate additional serine and threonine residues N-terminal to S45 (28). Phosphorylated β-catenin is then recognized by the F-box-containing protein β-TrCP, which mediates ubiquitination and proteosomal degradation of β-catenin (29-31). Together, these proteins make up the so-called β-catenin destruction complex. As we shall see later, this complex plays a central role in the (de)regulation of intestinal homeostasis.

Under physiological conditions, continued destruction of β-catenin is interrupted following Wnt binding to Fz/LRP. How the destruction complex senses Wnts at the cell surface is not fully understood. It has been assumed that the adapter protein Dsh through its association with Fz and the GSK3β-binding protein, Frat, may participate in this process (32-34). Note however that recent genetic evidence excludes an essential requirement for Frat in Wnt signaling, since mice with deletions in all three Frat family members develop entirely

normally (35). In parallel, Wnts induce phosphorylation of the cytoplasmic tail of LRP, which allows docking of Axin to LRP (36). Recruitment of Axin to the membrane is thought to disrupt the destruction complex thereby releasing β-catenin. Lastly, it has been suggested that stabilization of β-catenin may be promoted by the protein phosphatase PP2A, which appears to dephosphorylate GSK3β substrates including β-catenin (37).

Once released from the destruction complex, β-catenin translocates to the nucleus where it associates with the Tcf family of transcription factors (Tcf1, Lef, Tcf3 and Tcf4) (38). Tcfs function by targeting β-catenin to specific DNA elements found in promoters and enhancers of target genes (39;40). In turn, β-catenin recruits a number of nuclear factors responsible for transactivating Tcf target genes. Two of these factors include the histone acetylase CBP/p300 and the SWI/SNF component BRG1 (41-43). Activation of target genes also depends on the nuclear proteins Legless and Pygopus (44-46). It has been proposed that Legless and Pygopus are involved in directly activating transcription, possibly by recruiting chromatin remodelling factors. Legless and Pygopus may also function by transporting β-catenin to the nucleus (47). Finally it is worth noting that in the absence of nuclear β-catenin or when nuclear β-catenin is sequestered by factors such as ICAT and Chibby (48;49), Tcfs associate with general transcriptional repressors like Groucho (50;51). The latter silence target genes, in part, by recruiting histone deacetylases (HDACs), which render chromatin structure inaccessible to the basal transcriptional machinery. For an overview of the canonical Wnt pathway see Figure 1.

Wnt signaling in and the origin of intestinal epithelial cells

The intestinal epithelium originates from embryonic endoderm, which in turn stems from pluripotent epiblast cells at the onset of gastrulation (E6.0 in mice). During this stage, epiblast cells committed to form definitive endoderm ingress through the primitive streak displacing visceral endoderm. The first endodermal cells to travel through the primitive streak populate the anterior end of the embryo, whereas endoderm leaving at later stages colonizes more posterior regions. From E7.5-E9.5, the endodermal lining covering the mesoderm and ectoderm undergoes a series of invaginations initiated at the anterior and posterior ends of the embryo resulting in the formation of a proper gut tube (Figure 2). At this stage, the primitive gut is composed of a uniform layer of cuboidal endodermal cells surrounded by splanchnic mesoderm. The intestine along with the other organs derived from endoderm only become morphologically evident during a patterning phase (E9.5-14.5) in which the primordial gut is subdivided and reshaped along the anterior-posterior axis (Figure 2). For a thorough treatment of gut development see (52;53) .

The earliest role attributed to Wnt signaling during gut development was initially uncovered in ascidian embryos, where β-catenin was found to be essential for endoderm formation (54). Through gene targeting experiments, Kemler and colleagues showed that this function of β-catenin is evolutionarily conserved in mice (55). Ablation of β-catenin specifically in the node, notochord and anterior primitive streak abrogated definitive endoderm formation. Moreover, analysis of chimeric embryos showed that β-catenin-mutant

cells of the endodermal layer were unable to form endoderm but rather differentiated into precardiac mesoderm.

How β-catenin promotes definitive endoderm formation is unclear. Recent data has suggested that in endodermal cells, β-catenin may not necessarily act through Tcf factors. Indeed, Sinner et al. have proposed that in frogs, β-catenin drives the expression of endoderm specific target genes by physically associating with Sox17, an HMG box transcription factor, related to Tcfs (56). Given that in zebrafish and mice Sox17 also plays a role in the formation of definitive endoderm (57;58), it will be interesting to test whether the Sox17/β-catenin complex may represent a generalized mechanism for promoting endoderm specification. Another unanswered question raised by these findings regards the identity of the Wnt(s) stimulating β-catenin in the endoderm. In mice, Wnt3 is a possible candidate, since in Wnt3-mutant embryos, the epiblast remains undifferentiated while the primitive streak does not form. Moreover, the expression of both mesodermal and definitive endodermal markers is abolished (59).

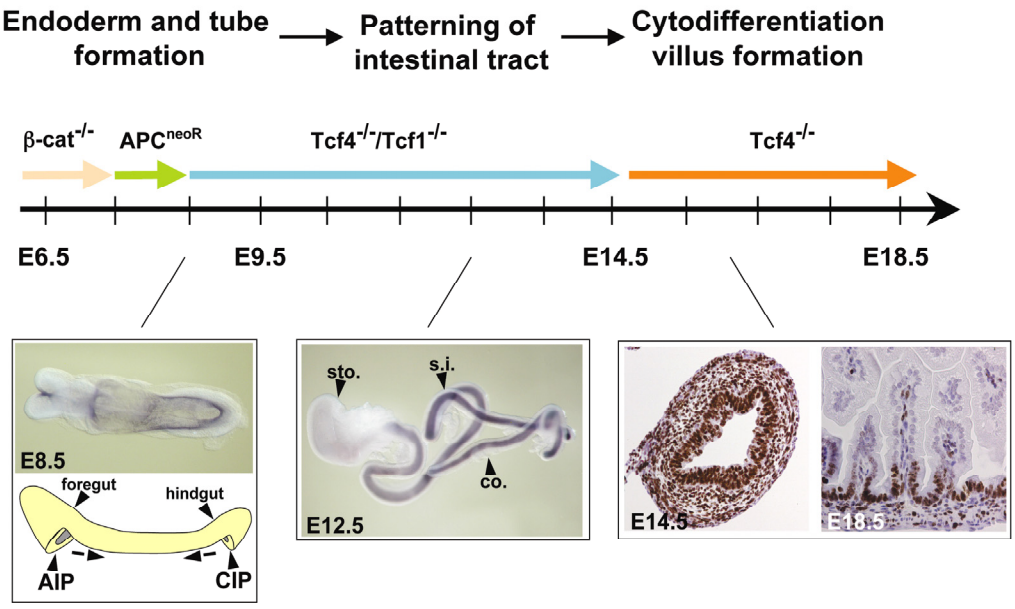

Figure 2. Time-line of intestinal development in the mouse.
Definitive endodermal cells are specified at E6.0 during gastrulation. The first panel from the left shows the bottom view of an E8.5 embryo, along with a schematic representation of the primitive gut. At E8.5 endodermal tube formation is initiated by folding (depicted by arrows) of the endodermal lining at the anterior and posterior ends creating anterior and caudal intestinal portals (AIP and CIP). The endodermal lining is stained with a probe recognizing *Foxa1*. At later stages (E9.5-E14.5) the primitive gut tube is patterned along the anterior-posterior axis. The expression of specific intestinal markers first appears in the hindgut at E9.0 (168;169).The second panel from the left shows a whole mount preparation of the entire gastro-intestinal tract (E12.5) stained for the intestinal marker *Villin*. Villus formation and cytodifferentiation (formation of enterocytes, goblet cells, enteroendocrine cells and Paneth cells) is initiated at E14.5. In the third panel from the left, sections from the small intestine were stained for the proliferation marker Ki67. Mutations associated with the Wnt pathway affect gut development at various stages (see text). Figure adapted from (53)

We have recently shown that Wnt signaling is required for gut tube formation (60). During this stage (E8.5), *in situ* hybridisation analysis revealed overlapping expression of Tcf4 and Tcf1in the hindgut. Simultaneous disruption of both genes led to severe defects in the formation of the hindgut and associated loss of expression of endodermal markers. This phenotype implies the existence of a Wnt source at the posterior end of the embryo, which would promote morphogenesis of the hindgut. A similar mechanism is utilized to drive posterior paraxial mesoderm and somite formation. In this case, Wnt3a expression in the presomitic mesoderm of the tailbud activates Lef and Tcf1 (61). Anterior tube formation may also depend on the activity of Wnt signaling components. Analysis of APC hypomorphic mutant mice (APC^{neoR}) has shown that expression of APC in the endoderm is required for the involuting movements, which generate the foregut pocket (see AIP in Figure 2) (62). The foregut defects in APC hypomorphs may result from the increased β-catenin/Tcf transcriptional activity in endodermal cells or may be ascribed to an alternative role for APC in cell migration.

Our analysis of Tcf4/Tcf1 mutant embryos at later stages also revealed malformations of the gastrointestinal tract consistent with both factors playing a role in patterning the gut (60). As could be expected from the early defects in hindgut formation, the intestine of $Tcf4^{-/-}$ /$Tcf1^{-/-}$ embryos is severely truncated. However, closer inspection uncovered anterior transformations at the stomach-duodenal junction. Expression analysis using specific markers of stomach and intestine revealed duplications of the stomach, suggesting that Tcf4 and Tcf1 promote an "intestinal" fate within the primitive gut and in their absence more anterior regions of the gut are expanded. Evidence supporting this interpretation was recently provided by Hogan and co-workers (63) who showed that when a constitutively active form of β-catenin is missexpressed in the lung endoderm, these cells turn on genes normally restricted to the intestine, implying once again that Wnt signals instruct endodermal cells to become intestine as opposed to other endodermal lineages.

Wnt signaling and adult intestinal homeostasis

Once the basic structure of the intestinal tract is laid out, differentiation along the radial axis may take place (see Figure 2 and 3). During this process the epithelium of the small intestine is remodelled to form characteristic finger-like projections (villi) and deep invaginations termed crypts. Similar events take place in the colon, where crypts form but where a flat surface epithelium exists instead of villi. These events coincide with the compartmentalization and cyto-differentiation of the epithelium. The intervillus regions of the fetal intestine, which are replaced by crypts in the first weeks after birth, are lined with highly proliferative progenitor cells. These transit-amplifying cells give rise to two differentiated cell lineages (ie. the absorbtive enterocytes and secretory cells). The secretory lineage can be further subdivided into mucus-secreting goblet cells, hormone-secreting enteroendocrine cells and bactericidal Paneth cells. Maturation of progenitor cells coincides with upward migration. Upon reaching the tips of the villi or the surface epithelium of the colon, the differentiated cells undergo apoptosis and are shed into the lumen. One exception

to this rule is the Paneth cell, which is generated from a progenitor migrating downwards toward the crypt base. The self-renewing capacity of the intestine depends on the existence of stem cells (64). Classical labelling experiments have shown that in the small intestine stem cells reside just above the Paneth cell compartment, while in the colon they occupy the first cell position at the crypt bottom.

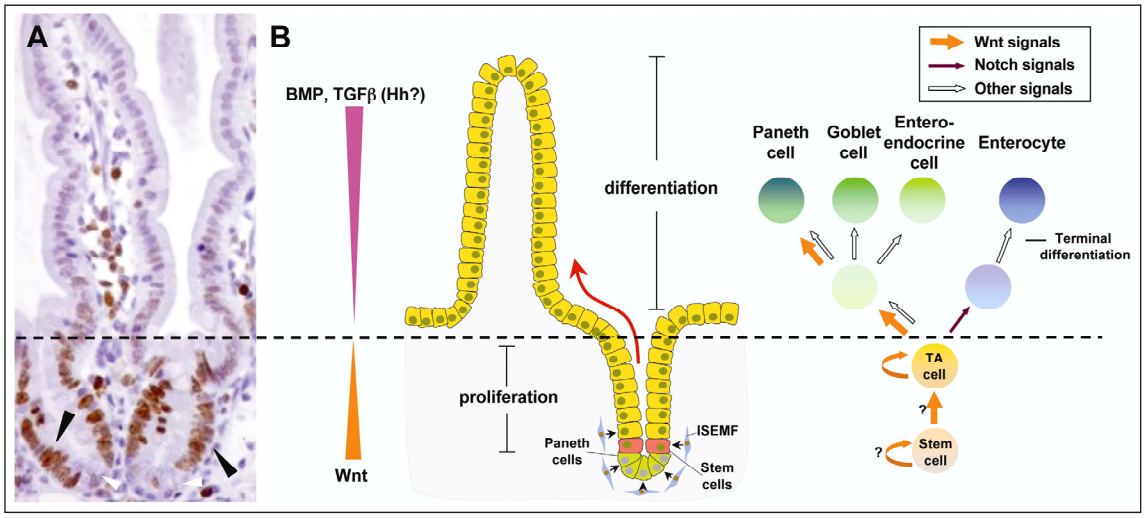

Figure 3. Adult intestinal homeostasis.
Panel A and B. Schematic representation and section of the crypt-villus unit in the mature small intestine. Proliferative cells reside in the crypts, while differentiated cells occupy the villus. Crypt progenitors migrate up (red arrow) the crypt-villus axis before shedding into the lumen. The process of epithelial renewal takes 3-6 days and is ensured by a small number of asymmetrically dividing stem cells at the bottom of the crypts. Wnt signaling in the adult intestine promotes proliferation of progenitor or transit-amplifying (TA) cells, as well as, commitment towards secretory lineages Wnt signaling may also drive terminal differentiation of certain secretory lineages (see text). Although it is commonly believed that Wnt signaling may promote proliferation and/or differentiation of intestinal stem cells, there is no evidence, which formally proves this (see arrows with question marks). In panel A, black arrowheads indicate Ki67 positive transit-amplifying cells, while white arrowheads indicate the Paneth cell compartment.

There are now several lines of *in vivo* evidence, which show that normal proliferation of the transit-amplifying cells is entirely dependent on continual stimulation of the Wnt pathway. Firstly, removal of Tcf4, β-catenin or overexpression of the Wnt inhibitor Dkk-1 results in a severe loss of proliferative epithelial cells in both the fetal and adult intestine (65-68). Cell cycle arrest is also observed in CRC cell lines in which β-catenin/Tcf activity is blocked either through expression of dominant negative Tcf4 or knockdown of β-catenin (69;70). Consistent with these results, mutations in the negative regulator of Wnt signaling APC, or overexpression of oncogenic forms of β-catenin result in hyperproliferation of the epithelium (9;71-73). Lastly, progenitors located at the bottom of the crypts accumulate nuclear β-catenin implying that these cells respond to Wnt stimulation (69). Although these studies confirm the strong link between Wnt signalling and maintenance of transit-amplifying cells, it should be noted that virtually no evidence exists to draw a similar link between Wnt signals and stem cells (Figure 3). Part of the difficulty in tackling this issue is related to our lack of reliable markers of intestinal stem cells. Besides proliferation, we may also consider the accumulating evidence implying an additional function for Wnt signaling in driving the

differentiation of secretory lineages. Indeed, blocking active Wnt signaling *in vivo* results in a reduction or absence of goblet, enteroendocrine and Paneth cells, while enterocytes appear spared (65;66;68).

Supported by these findings, a model can be proposed whereby transit-amplifying cells responding to a source of Wnts at the crypt bottom proliferate and concomitantly commit themselves to the secretory lineage. As these progenitors move up the crypt and further away from the Wnt source, Tcf/β-catenin activity is turned off, thus favouring cell-cycle arrest and terminal differentiation. This simplistic view overlooks a number of issues. In our discussion, below we shall highlight four major questions: What is the genetic program regulated by Tcf/β-catenin in crypt progenitors? Where and what is the Wnt source? How does Wnt signaling regulate secretory cell lineage commitment? And finally, how is Wnt signaling turned off?

Tcf/β-catenin target genes

Most studies aimed at identifying Tcf/β-catenin target genes (for simplicity the term target gene here refers to either direct or indirect Wnt-responsive genes) in intestinal cells have made use of systems in which β-catenin is constitutively activated such as in CRC cell lines (see Table I for a selected list of Tcf target genes). Consequently as we shall see later, the majority of Tcf/β-catenin target genes have been associated with various processes important for tumorigenesis (ie cellular proliferation, survival and motility). Given that many of these genes are also expressed in normal crypt progenitor cells (69), efforts are now being undertaken, through classical loss or gain of function experiments in mice, to test their function during intestinal development and homeostasis. So far, however, only a limited number Tcf/β-catenin targets have been tested *in vivo*.

The proliferative effects of Wnt signaling on crypt progenitors have, for some time now, been linked to cell cycle regulators such as c-Myc and cyclin D1 (74-76). Both factors are overexpressed in colorectal tumors and blocking expression of either gene inhibits proliferation in CRC cell lines (69;77;78) . However, cyclin D1 null mice do not appear to exhibit any abnormalities in the intestine, other than a modest reduction in the propensity to develop polyps when crossed with the APC^{min} mice (79). This observation suggests that other cyclin Ds may be more relevant downstream β-catenin/Tcf target genes. Along these lines, our own observations have shown that cyclin D2 is an early downstream Tcf4 target in the fetal gut (Gregorieff and Clevers, unpublished data). Moreover, microarray studies have shown that cyclin D1 levels do not appear to be affected when conditionally deleting APC (73). C-Myc has been confirmed as a Tcf/β-catenin target *in vivo* (68;80), although its precise function in normal epithelial cells remains to be clarified. In the bone marrow, recent evidence suggests that c-Myc promotes the release of hemopoietic stem cells from the stem cell niche by regulating the expression levels of adhesion molecules (81). In the skin, ectopic expression of c-Myc diverts epidermal stem cells to a sebaceous gland fate at the expense of hair follicles (82). Whether c-Myc performs similar functions in the intestine will need to be examined by a conditional knock-out approach.

Another Tcf/β-catenin target gene, which has been implicated in promoting proliferation is Id2 (69;83). The Id proteins represent a family of naturally occurring inhibitors of basic helix-loop-helix transcription (bHLH) factors, and function in many circumstances to prevent differentiation (84). In particular, Id2 is highly abundant in several cancer types and when forcibly expressed in colon cancer cell lines Id2 has been shown to increase anchorage-independent survival (83). Recent *in vivo* evidence, on the contrary, suggests that Id2 may have a completely different role in crypt progenitor cells (85). In the Id2 knockout intestines, differentiation of endoderm is impaired during the late fetal stages (E18.5). Consequently the villi in several areas appear replaced by multilayered, undifferentiated endoderm. These areas of pseudostratified epithelium later develop into dysplastic and metaplastic tumours exhibiting high levels of nuclear β-catenin. Interestingly, these lesions also show a loss of Paneth cells and enteroendocrine cells and increased numbers of Goblet cells.

Expression profiling has also identified genes implicated in many other processes besides the control of proliferation and/or differentiation. The tyrosine kinase receptors EphB2 and EphB3 and their ligand ephrin B1 illustrate this point (86). Consistent with their well-known roles in cell sorting in various tissues, these receptor/ligands pairs are expressed in an inverse gradient along the crypt-villus axis, with EphB2 and -B3 high in crypt cells and their ligand ephrin-B1 predominating in the villi. This expression pattern is tightly regulated both *in vitro* and *in vivo* by Tcf/β-catenin. *In vivo* confirmation of the importance of these molecules came from the analysis of $EphB2^{-/-}B3^{-/-}$ KO. In these mice, proliferative and differentiated cell populations intermingle. Furthermore in $EphB3^{-/-}$ mice, Paneth cells no longer home to the crypt bottom, but rather scatter along crypts and villi. Thus, a Wnt signalling gradient controls cell positioning along the crypt-villus axis through regulation of EphB2 and EphB3 gene expression.

The functional characterization of Tcf/β-catenin target genes will continue to be a major focus of interest for the coming years. We shall return to this issue in the context of colon carcinogenesis.

The Wnt source

The exact location or identity of the Wnts that drive proliferation is unclear. Nevertheless it is believed that mesenchymal cells or, more specifically, intestinal subepithelial myofibroblasts (ISEMFs), immediately adjacent to crypt epithelial cells, are a source of Wnts (87;88). This notion is based in part on classic co-culture experiments, which have shown that these cells are able to simulate proliferation of epithelial cells (89) We recently tested this hypothesis by screening all 19 Wnts for expression in the adult intestine (Gregorieff and Clevers manuscript submitted). Through this approach we found several Wnts expressed in crypt epithelial cells, but so far none were detected in ISEMFs. Ablation of the Wnt genes associated with crypt epithelial cells will be required to test their function. Until then, if we are to assume that these Wnts drive proliferation, then the next obvious question is what regulates Wnt expression in the epithelium. Here once again we may have to turn to

ISEMFs. These cells are known to produce paracrine growth factors (90), which conceivably could activate Wnt gene expression in the epithelium. This idea however remains speculative.

On a related issue, genetic evidence in mice has identified two transcription factors, FoxL1 and Nkx2.3, involved in regulating growth signals emanating from the mesenchyme (91-93). Deletion of either gene in mesenchymal cells results in increased epithelial proliferation, suggesting that FoxL1 and Nkx2.3 normally play an inhibitory role. Kaestner and colleagues observed upregulation of the HSPGs, Syndecan1 and Perlecan, in FoxL1$^{-/-}$ mice (94). Although HSPGs have been implicated in stimulating Wnt signals (19;94), it remains to be tested whether these changes are a cause or an effect of the increased proliferation.

Cell lineage commitment

The disproportionate reduction in goblet, enteroendocrine and Paneth cell numbers, resulting from the ablation of Wnt signals, suggests a definite role for Wnts in specifying secretory lineages. Recently, some general rules for cell lineage commitment in the intestine have been uncovered. Precursors of all three secretory cell types express the bHLH factor, MATH1. Accordingly, MATH1 deficient mice lack goblet, enteroendocrine and Paneth cells but do produce enterocytes (95). The latter cells derive from progenitors expressing Hes1, based on the fact that Hes1$^{-/-}$ intestines display increased numbers of secretory cells at the expense of enterocytes (96). Interestingly, Hes1 transcription is activated by Notch signaling in other biological models (97), while Hes1 transcriptionally represses MATH1 expression (96;98). Together these findings suggest a model whereby commitment towards the enterocyte lineage would be favoured in cells with active Notch signaling, turning on Hes1 transcription. Inversely, in the absence of Notch signaling, MATH1 would be upregulated skewing the cells towards secretory lineages (Figure 3B).

Further commitment towards specific cell types depends on yet other transcription factors. For example, the activation of NGN3, BETA2, Pax4 and Pax6 is associated with enteroendocrine (sub-)lineages (99), while differentiation of goblet cells is influenced by KLF4 (100). Moreover, in ELF3$^{-/-}$ mice differentiation of absorptive and goblet cells is impaired (101).

The connection between Wnt signaling and any of these factors remains an open question. One putative link was suggested by the observation, as we have mentioned earlier, that ablation of the Wnt target gene and bHLH antagonist Id2, results in impaired production of secretory lineages (85). It is plausible that Id2 may mediate these effects by directly antagonizing the activity of certain bHLH trancription factors such as MATH1. Alternatively, Wnt signals may directly activate the expression of genes involved in cell lineage commitment. Although no evidence for this exists so far, expression profiling of mouse models displaying impaired Wnt signaling suggests that the final stages of maturation of secretory lineages may depend on active Wnts signals. In particular, we and others find that

the expression of Paneth cell markers, such as anti-microbial peptides (i.e. cryptdins and defensins), are directly stimulated by β-catenin/Tcf (102;103).

-*Counteracting Wnt signaling*

There are two non-mutually exclusive mechanisms, which could explain how the stimulatory effects of the Wnt cascade are turned off in the intestine. In one scenario, activation of Wnt signals would gradually and passively dissipate as progenitors migrate up along the crypt-villi axis in sites where canonical Wnts are limiting. On the other hand, a more active mechanism may be utilized, involving "negative" cross-talk between the Wnt pathway and other signaling pathways. As we shall discuss below the TGFβ and BMP cascades are associated with growth inhibition in the gut, and thus may represent examples of Wnt-counteracting pathways.

TGFβ signaling components are localized in differentiated epithelial cells, where they have well-documented growth suppressive effects (104). Furthermore, in both man and mouse, benign adenomas acquire invasive properties following the acquisition of inactivating mutations in the TGFβ-RII receptor or the intracellular signaling components Smad2 and 4 (105-107). Mice with germline mutations in Smad3 and the latent TGFβ binding protein 4 (LTBP-4) also develop colorectal cancer (108;109). Several groups have described mechanisms by which TGFβ signals could antagonize Wnt signaling in the intestine. One possible route may involve the alternative TGFβ effector and MAPKKK, TAKI. In *Caenorhabditis elegans* and mammalian cells activation of TAK1 stimulates the activity of the MAPK, NLK, which in turn, downregulates Tcf (110;111). Alternatively, Sasaki et al. have shown that TGFβ stimulation inhibits Tcf4/β-catenin transactivation of c-Myc via the ability of Smad3 to physically interact with β-catenin and thereby decouple Tcf4/β-catenin complexes (112).

Similar inhibitory functions have been attributed to BMPs. BMP2 and BMP4 are expressed in mature epithelial cells and villus mesenchyme, respectively. Moreover, both factors appear to activate their downstream signaling components SMAD1, 5 and 8 in the differentiated epithelium (113;114). Patients harbouring mutations in BMP signalling components suffer from juvenile polyposis syndrome (JPS), which is characterized by the formation of hamartomatous polyps throughout the gastrointestinal tract (115-117). Similar polyps are formed in the stomach and duodenum of Smad4 heterozygous mice. Insight into how these defects occur was recently provided by the generation of transgenic mice expressing the BMP inhibitor, Noggin, in the intestinal epithelium (113) and in mice in which the BMPR1A gene was conditionally deleted in the intestinal epithelium (118). In both cases, these mice develop lesions equivalent to those found in JPS. At the earliest stages in the development of these lesions, BMP inhibition results in *de novo* crypt formation combined with increased numbers of proliferative cells in normally differentiated compartments of the villi. Based on these observations, it appears that BMP signaling may restrict ectopic Wnt-mediated proliferation in the differentiated epithelial cells and thereby confine crypt formation to regions immediately adjacent to the muscularis. How BMPs would antagonize

Wnt signaling in the intestine still remains to be clarified. However, a tentative model has been proposed by He et al. (118), in which BMP4 somehow promotes PTEN activation in intestinal stem cells, which in turn would repress β-catenin/Tcf activity through the PI3 kinase-AKT pathway. These results await further confirmation.

Another class of signaling molecule, which may oppose the effects of Wnt signaling in the intestine are the Hedgehogs (Hh). The available evidence supporting such a role is somewhat conflicting. During chick and mouse, development Sonic hedgehog (Shh) and Indian hedgehog (Ihh) play multiple roles in patterning of the gastrointestinal tract (119-123). Both proteins have been implicated in the growth of upper-digestive tract tumours (124;125). Van den Brink et al. examined the role of Ihh signaling in the colonic epithelium (126). In the human colon, Ihh is uniquely expressed amongst non-proliferative cells of the surface epithelium. Accordingly, rats treated with cyclopamine, a small-molecule inhibitor of Hh signalling, displayed defects in enterocyte differentiation and an increase in the number of cycling cells per crypt. These authors also showed that Ihh signaling *in vitro* interferes directly with β-catenin/Tcf transcriptional activity. More recently, ectopic epithelial proliferation was also reported in mice transgenically expressing the pan-Hh inhibitor HIP in the intestinal epithelium (88). However, ablation of Hhs in mice by homologous recombination contradicts these results (122). Ihh deficient mice display a loss of enteric neurons and as a result develop dilated colons reminiscent of Hirschprung's disease; while in the small intestine, the number of cycling epithelial cells is reduced. Shh mutant mice show intestinal metaplasia in the stomach and duodenal stenosis.

Wnt signaling and colorectal cancer

In humans, sporadic and hereditary forms of colorectal cancer develop along a well-defined sequence of histopathological changes (127). The earliest lesions occurring in the colonic epithelium - aberrant crypt foci (ACF) - are characterized by dysplastic or hyperplastic crypts. Subsequent expansion of the ACF generates larger adenomas, which in turn may progress to carcinoma *in situ* and invasive adenocarcinomas. Because these lesions are easily identifiable, researchers have been able to characterize the genetic alterations associated with each stage (104;127)). The earliest mutations identified in the adenoma-to-carcinoma sequence alter the function of components of the Wnt pathway. Mutations in APC are responsible for an inherited form of CRC, termed familial adenomatous polyposis (FAP) (7;8). Moreover, the overwhelming majority (80%) of early adenomas from sporadic cases of CRC bear truncating mutations in APC (9). Some of the remaining cases of CRC result from mutations in β-catenin, and Axin2 (128-130). Below we shall discuss how activating mutations in the Wnt cascade confer upon cells a selective growth advantage, which allows for the initial expansion of the precancerous lesion.

Table I. List of β-catenin/Tcf target genes tested functionally *in vitro* or *in vivo*.

Gene	Type	LOF/GOF	Ref.
c-Myc	-bHLH transcription factor	-knock-down blocks proliferation	(69;74)
Cyclin D1	-cell cycle regulator	-cyclinD1$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	(75;76;79)
Id2	-inhibitor of bHLH transcription factors	-Id2$^{-/-}$ develop tumors and show impaired differentiation	(83;85)
ITF-2	- bHLH transcription factor	-overexpression promotes neoplastic transformation	(155)
Tcf1	-Wnt signaling	-Tcf1$^{-/-}$/ APC$^{min/+}$ show increased polyp burden	(145)
PPARδ	-ligand-activated transcription factors	-PPARδ$^{-/-}$/ APC$^{min/+}$ show increased polyp burden -treatment with PPARδ agonist, GW501516, increases number and size of polyps in APC$^{min/+}$	(142;156-158)
COX-2	-prostaglandin pathway	-COX-2$^{-/-}$/ APC$^{\Delta 716/+}$ show reduced polyp burden	(143;144;159)
HDAC2	-histone deactylase	-treatment with HDAC2 inhibitor, valproic acid, reduces polyp number in APC$^{min/+}$ mice	(160)
FGF18	-growth factor	-knock-down suppresses growth of CRC cells	(140)
FGF20	-growth factor	-knock-down suppresses anchorage-independent growth	(161)
Endothelin	-growth factor	-rescues growth arrest and apoptosis resulting from blocking β-catenin	(162)
Gastrin	-gastrointestinal growth factor and hormone	-Gastrin$^{-/-}$/ APC$^{min/+}$ show reduced polyp burden	(139)
BAMBI	-BMP and activin membrane-bound inhibitor	-overexpression blocks TGFβ-mediated growth inhibition	(163)
MMP7/ Matrilysin	-ECM protease	-MMP7$^{-/-}$/ APC$^{min/+}$ show reduced polyp burden	(81)
Nr-CAM	-adhesion	-overexpression increases cellular motility	(137)
Mdr1	-ABC transporter	-Mdr$^{-/-}$/ APC$^{min/+}$ show reduced polyp burden	(164;165)
ENC1	-BTB/Kelch protein family member	-overexpression increases growth rate in CRC cells	(166)
APCDD1	-unknown	-knockdown inhibits cell/tumor growth	(167)

Consequences of hyper-active Wnt signaling

The immediate consequences of mutations in APC and β-catenin are well understood. β-catenin mutations disrupt the CK1/GSK3β phosphorylation sites at the N-terminus of the protein (128;131). Consequently, mutant β-catenin is no longer recognized by β-TrCP and becomes stabilized. In turn, mutant β-catenin is free to enter the nucleus and constitutively activate transcription through Tcfs. Equivalent effects result from APC inactivation. Truncation of APC removes repetitive elements within the protein responsible for binding to β-catenin and Axin (132). As a result, GSK3β phosphorylation and subsequent degradation of β-catenin is severely impaired. Frameshift mutations in Axin2 eliminate its DIX domain required for homo-oligomerization. Although expression of mutant Axin2 in cells results in increased β-catenin accumulation, it is unkown how mutant Axin2 interferes with the destruction complex (129).

Besides affecting the function of the destruction complex, mutations in APC have been proposed to disrupt its ability to regulate β-catenin function in the nucleus (132;133). For example, APC contains both nuclear export and import signals which allow it to act as a nuclear-cytoplasmic shuttle. Once in the nucleus APC promotes export of β-catenin and thereby deactivation of Tcf-mediated transcription, a property lost by mutation of APC (134;135). Alternatively, by associating with the transcriptional repressor CtBP, APC may also interfere with the formation of β-catenin/Tcf complexes (136). Whether these additional pathways regulating β-catenin activity play a significant role in neoplastic transformation remains to be determined.

How does constitutive β-catenin/Tcf transcriptional activity promote adenoma formation? As we first discussed in the context of homeostasis, the Wnt pathway normally promotes proliferation of progenitor cells. It is silenced when these cells exit the crypt compartment. In general terms, we may say that adenomas result from the unabated expansion of cells, which have adopted a crypt progenitor-phenotype. Consequently, the genes activated by aberrant β-catenin/Tcf activity in CRC cells simply reflect the normal genetic program of crypt progenitors (69).The identity and function of these target genes has been a hot topic in recent years. Today, the number of candidate β-catenin/Tcf effector genes has exploded and includes genes which may intervene in the cell cycle (c-myc, cyclinD1), tumor cell migration (eg. MMPs, Nr-CAM) (81;137) , survival (eg. Survivin) (138), growth (eg. FGF18, Gastrin) (139;140), as well as angiogenesis (VEGF) (141) and prostaglandin signaling (eg. COX-2, PPARδ) (142-144). A complete description of all putative Tcf target genes identified so far would be well beyond the scope of this review. Instead we refer the reader to Table I, which highlights target genes that have been tested functionally in CRC cells.

Of particular relevance to this review is the observation that aberrant β-catenin/Tcf activity also leads to transcriptional upregulation of components of the Wnt signaling pathway proper. In colon cancer cells, both Tcf1 and Lef are strongly upregulated through direct activation by Tcf4. Genetic evidence in mice has shown that Tcf1 acts as tumor

suppressor (145). Tcf1 knockout mice display a predisposition towards developing spontaneous intestinal adenomas and polyp counts are greatly increased in $APC^{min/+}$ mice lacking Tcf1. One untested hypothesis put forward to explain these results is the suggestion that in colon cancer cells Tcf4 promotes expression of dominant-negative isoforms of Tcf1, lacking the β-catenin interaction domain. As such, activation of Tcf1 expression would constitute a negative feedback loop involved in inhibiting high levels of β-catenin/Tcf activity. The effects of upregulating Lef in tumour cells have not been tested *in vivo*. However, Lef is likely to play a positive role in tumorigenesis based on the observation that Tcf4 specifically activates transcription of full-length Lef isoforms capable of interacting with β-catenin (146). In addition, Lef has been shown to harbour distinct biochemical properties when compared to Tcf4. For instance, Lef, contrary to Tcf4, appears to be refractory to the inhibitory effects of a TGFβ-Smad3 pathway (112).

Another β-catenin/Tcf target gene and Wnt signaling component relevant to cancer is Axin2. In normal cells, as part of a negative feedback mechanism, Axin2 is upregulated following Wnt stimulation (147-149). As we have described earlier, this apparently attenuates excessive Wnt stimulation since inactivating mutations in Axin2 promote tumorigenesis. Upregulating Axin2 in adenomas may also serve to suppress the effects of aberrant β-catenin signaling. This idea is supported by the finding that overexpression of Axin in CRC cell lines bearing mutations in APC (but not β-catenin) downregulates β-catenin levels (150). The significance of these observations awaits further *in vivo* confirmation.

New players in Wnt pathway-driven colorectal cancer?

Given the predominant role of the Wnt pathway in CRC and many other types of cancer, several laboratories have shifted their attention on other Wnt signaling components besides the usual culprits such as APC and β-catenin. Recently, two groups have documented, in a high percentage of human colorectal adenomas and aberrant crypt foci, epigenetic silencing of the genes encoding for SFRPs (151;152). Suzuki et al. followed up on these initial observations by testing the impact of expressing SFRPs in CRC cell lines. Transfection of SFRP1, 2 and 5 in HCT116 and SW480 cells decreased β-catenin levels and transcriptional activity and resulted in growth inhibition and apoptosis. However, in similar experiments performed by Bafico et al., SFRP1 only had inhibitory effects on engineered HCT116 cells containing a single wild type β-catenin allele, whereas parental HCT116 cells with both wild type and mutant alleles or HCT116 cells containing only a mutant allele were insensitive to SFRP1 (153). Despite these discrepancies both groups show that HCT116 produce several Wnts and that treatment with SFRPs blocks autocrine Wnt-induced proliferation. Taking into account the results from Bafico et al., it is more likely that silencing of SFRPs would only provide a growth advantage before mutations in APC and β-catenin have occurred. At later stages of tumorigenesis when cancer cells constitutively express high levels of β-catenin, disrupting Wnt function would most likely be inconsequential.

Concluding remarks

As we have highlighted in this article, the intestinal epithelium provides an attractive system to study how Wnt signaling regulates cellular growth and differentiation. Current evidence validates the Wnt cascade -in particular the β-catenin/Tcf4 complex- as a target for therapeutic strategies in the treatment of CRC. Breaching the interaction between β-catenin and Tcf in cancers using small organic molecules will be a hard nut to crack. Yet, some promising results have recently been reported by Shivdasani and colleagues (154). The challenge in the long term will be to translate our increasing knowledge of the biochemical and functional features of the Wnt pathway into effective therapeutic strategies to combat cancer.

Outline of the thesis

As was discussed in the introduction, the intestine has served as a useful model system to investigate the biological role of the Wnt pathway. Over the years, a great deal of attention has been directed towards unraveling the link between mutations in Wnt signaling components and initiation of colon carcinogenesis. The first major breakthrough came with the discovery that APC and β-catenin mutations result in increased Tcf-driven transcriptional activation. Through the identification of Tcf-mediated target genes it later became clear that the Wnt pathway controls a genetic program involved in maintaining intestinal epithelial cells in a highly proliferative state. These observations provided a basic understanding of how activation of the Wnt pathway confers upon colon cancer cells a growth advantage, as well as revealed the importance of Wnt signaling during gut homeostasis. However having said this, numerous questions regarding Wnt signaling and its role in the intestine have largely been left unexplored. This thesis attempts to address some of these issues.

One of the objectives was to investigate how Wnt signaling affects early development of the gut. In Chapter 2, we present evidence which shows how in gastrulating embryos, Tcf4 and Tcf1 act redundantly to promote expansion of the primitive hindgut. We also show that at later stages loss of both Tcf4 and Tcf1 results in patterning defects of the gastro-intestinal tract. The goal of Chapter 3 was to determine the identity and localization of the Wnt signaling components expressed in the fetal and adult intestine. To tackle this issue we performed an extensive *in situ* hybridization screen of all known Wnt ligands, receptors, antagonists, as well as Tcf factors. This analysis allowed us to uncover factors putatively involved in regulating both canonical and non-canonical Wnt signals. In Chapters 4 and 5, with the prior knowledge that Tcf4 is required to maintain proliferation of intestinal epithelial cells, we sought-out to uncover genes regulated by Tcf4 during late fetal development. Paradoxically, this approach revealed that Wnt signaling regulates genes normally expressed in secretory lineages (ie. goblet cells, Paneth cells and enteroendocrine cells). These observations indicate that Wnt signaling promotes proliferation of progenitor cells and concomitantly favors their differentiation.

CHAPTER 2

Hindgut defects and transformation of the gastro-intestinal tract in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos

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Hindgut defects and transformation of the gastro-intestinal tract in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos

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Abstract

Wnt signalling plays a critical role in both initiating and patterning of the anterior-posterior (A-P) axis during development. Wnts exert their biological effects, in part, by activating specific target genes through members of the TCF/LEF family of transcription factors. To gain new insight into the role of T-cell factors (or Tcf's) during development we analysed *Tcf4* and *Tcf1* compound null embryos. These mutants showed severe caudal truncations, as well as duplications of the neural tube. Unlike other mutations affecting Wnt signalling, paraxial mesoderm formation was not impaired and early caudal markers, such as *T*, were unaffected. Analysis of endodermal markers uncovered early and specific defects in hindgut expansion and later an anterior transformation of the gastro-intestinal tract. Our results reveal a novel role for Wnt signalling in early gut morphogenesis and suggest that specific Wnt-driven patterning events are determined by the unique tissue distribution of *Tcf/Lef* family members.

Introduction

During mouse development, the anterior-posterior (A-P) axis becomes morphologically evident at the onset of gastrulation. At this point, the epiblast cell layer at the posterior end of the embryo forms a structure known as the primitive streak. Cells at the streak delaminate and migrate forward and laterally to form both mesodermal and endodermal layers. As gastrulation proceeds at the posterior end, body structures such as head, trunk, limbs and tail are specified along the A-P axis. This process of patterning and later organogenesis depends on a complex network of instructive signals, ultimately determining whether cells within the developing embryo proliferate, differentiate, or die.

Amongst the most important regulators of cell fate decisions during both formation and patterning of the A-P axis are members of the Wnt/Wg family of secreted factors. Wnts make up a large family of cystein-rich glycoproteins that activate signalling cascades that induce cytoplasmic responses and/or transcription of target genes [for reviews see (13;170-172). The latter depends on cytoplasmic accumulation of β-catenin and its translocation to the nucleus where it associates with the TCF/LEF family members (comprised of TCF1, LEF, TCF3, TCF4). Formation of this bipartite complex provides a template for the recruitment of additional transcriptional activators that finally turns on expression of specific target genes. Importantly, in the absence of Wnts, TCF/LEF proteins can actively block transcription by recruiting general repressors like Groucho and CtBP (173;174). In this way TCF/LEF proteins act as molecular switches that control cell fate decisions.

Perhaps the first indication that the Wnt/β-catenin/TCF pathway was required for determining the A-P axis came from microinjection experiments conducted in *Xenopus* embryos. Ectopic expression of Wnts, for example, induces axis duplications and can rescue UV irradiated embryos (175;176). In addition, gene-targeting experiments in mice have provided a wealth of information confirming an essential role for Wnt signalling in axis formation. Mutations disrupting Wnt signalling, such as *Wnt3* and *β-catenin* knock-outs, completely impair formation of the primitive streak and resulting embryos remain as egg cylinders lacking posterior specification (59;177). On the other hand, mutations in *Axin* and *APC* which lead to increased β-catenin/TCF-mediated transcription, cause axis duplications similar to those observed in *Xenopus* (178). Similarly, ablation of *Tcf3* results in duplications of the node and notochord, as well as upregulation of axial markers, such as *Foxa2*, implying that *Tcf3* acts primarily as a transcriptional repressor involved in restricting AP axis induction (179).

Deletion of other components of the Wnt cascade has shown that Wnt signals are also required for patterning and/or expansion of the mouse embryonic axis once it has formed. Mutations in *Wnt1* lead to deletion of the midbrain and cerebellar component of the hindbrain (180). *Dkk*$^{-/-}$ embryos display a disruption of forebrain and cephalic neural crest-derived tissues as well as limb morphogenesis defects (181). *Wnt3a*$^{-/-}$ and *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ embryos show severe caudal truncations of the embryonic axis, resulting from an early loss of mesodermal

structures (ie. somites) (61;182;183). The absence of *Wnt5a* results in a general shortening of the embryonic axis, including limbs (184). Finally, loss of *LRP6*, an essential Wnt-coreceptor, recapitulates many of the phenotypes described above, with disrupted patterning in the brain, limbs, neural tube and tail (21).

Besides the phenotype of *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ and *Tcf3*$^{-/-}$ embryos described above, limited information is available about the function of other *Tcf/Lef* genes during early mouse development. *Lef*$^{-/-}$ mice die perinatally lacking teeth, hair follicles and mammary glands (185). Further analysis has also demonstrated impaired hippocampus development and generation of dentate gyrus granule cells in *Lef* deficient mice (186). *Tcf1* homozygous null mice are viable but demonstrate an early blockage in thymocyte differentiation, as well as increased susceptibility to form mammary gland and intestinal neoplasms (145;187). *Tcf4*$^{-/-}$ mice die at birth due to a loss of epithelial stem cells in the small intestine (66). In order to gain further insight into the function of *Tcf/Lef* genes during morphogenesis, we generated compound *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ mice. These mutants display profound caudal truncations similar to *Wnt3a*$^{-/-}$ and *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, although unlike the latter, *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos retain paraxial mesoderm. Rather, the primary patterning defect appears to result from aberrant hindgut expansion, ultimately leading to homeotic shifts of the fetal gastro-intestinal tract.

Results

Tcf4$^{-/-}$/*Tcf1*$^{-/-}$ mutants show posterior truncations.

To study the consequences of inactivating both *Tcf1* and *Tcf4* during embryonic development we crossed *Tcf4*$^{+/-}$ and *Tcf1*$^{-/-}$ mice. Although smaller than wild-type counterparts, *Tcf4*$^{+/-}$/*Tcf1*$^{-/-}$ mice appeared normal. Compound homozygous null embryos, however, did not survive past gestation. Viable double mutants were obtained at the expected frequency until E14.5. Examination of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos at this stage showed major abnormalities in the development of caudal structures while anterior structures were spared. As shown in Figure 1, *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ mutants lacked hindlimbs, posterior body and tail. Although formation of hindlimbs was affected, forelimbs were normal suggesting that the loss of hindlimbs reflect a general patterning defect, rather than a specific problem in limb development. In more severely truncated embryos internal organs were exposed indicating a failure of the abdominal wall to properly develop (Figure 1C and D). Hematoxylin/eosin stainings of sections of the specimen depicted in Figure 1B revealed that most internal structures, including lungs, heart, pancreas, and liver were clearly distinguished and histologically appeared normal (Figure 1F and data not shown). Even more caudal structures such as kidneys and mesonephric ducts had formed and differentiated. This may imply that *Tcf4* and *Tcf1* are not required for early morphogenesis of these organs. Closer examination of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ mutants, however, showed a complete absence of the genital tubercle, hindgut and only a remnant of the urogenital sinus, as well as signs of aberrant branching of neural tube (Figure 1D, F and below).

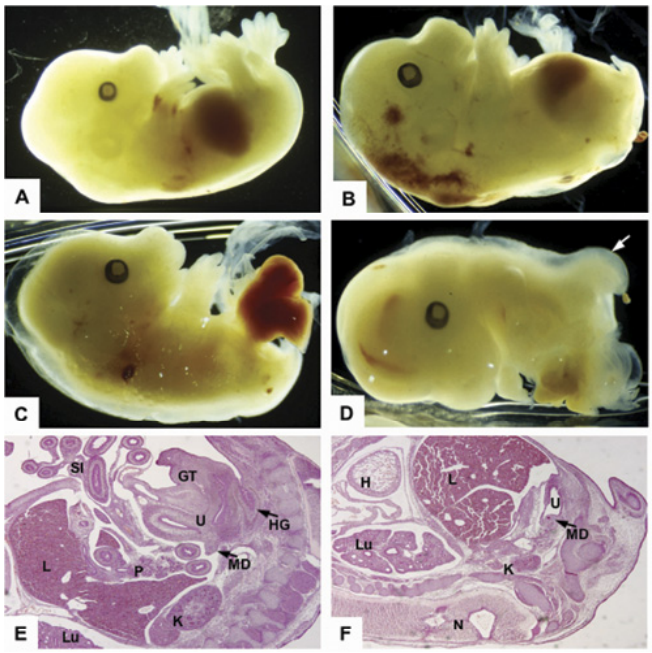

Figure 1. *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos show severe caudal truncations.
Lateral views of E14.5 embryos. (A) *Tcf4*$^{+/-}$/*Tcf1*$^{-/-}$ and (B-D) *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ littermates. Panel B shows vestigial hindlimbs and the absence of a tail in mutant embryos. In more severe cases as depicted in panels C and D internal organs such as the gut and liver are exposed. In several instances the neural tube branched out in mutant embryos as shown in D and F (see arrow). Panels E and F represent hematoxylin and eosin stainings of sections of embryos depicted in A and B respectively. Various structures are identified as follows: (H) heart, (Lu) lungs, (L) liver, (K) kidneys, (U) urogenital sinus, (MD) mesonephric duct, (GT) genital tubercle, (SI) small intestine, (HG) hindgut, and (N) neural tube.

Duplications of the neural tube in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

The posterior truncations observed in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos were reminiscent of *Wnt3a*$^{-/-}$ mutants. Analysis of these mice, as well as *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, revealed an early loss of paraxial mesoderm and concomitant formation of ectopic neural tubes (61;188). To characterize the fate of paraxial mesoderm vs. neural tissue in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, we began our analysis by examining the expression of the neural tube marker *Wnt-1* in E10.5 embryos. As shown by whole-mount *in situ* hybridization, *Wnt-1* expression along the anterior portion of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos was comparable to that of normal littermates (Figure 2). Towards the posterior end, ectopic *Wnt-1* expression was detected, reflecting bifurcations or splitting of the neural tube (Figure 2D, see arrow). Sectioning through the mutant embryos confirmed the occurrence of duplications of the neural tube (Figure 2E).

Paraxial mesoderm formation is unaffected in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

To follow the fate of paraxial mesoderm we employed three somitic markers, *Paraxis*, *Pax1* and *Myf5*. Detection of *Paraxis* transcripts, normally present in sclerotome and dermamyotome compartments, clearly distinguished the presence of somitic tissue along the entire A-P axis of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos (Figure 3B). Note however that the somites at the caudal end did appear disorganized. Even in a specimen displaying a more severe truncation, the sclerotome marker, *Pax1,* revealed that despite being smaller and less well defined, somites were present (Figure 3D). Finally, we tested the myogenic marker, *Myf5,* and found once again that caudal somites had formed (Figure 3F). The maintenance of somitic markers was in stark contrast to the previously described *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ phenotype, in which case *Pax1* expression was completely absent below the forelimbs (61). To confirm the differences between the two crosses we generated *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ embryos and verified the expression of *Paraxis* in these mutants (data not shown). As expected, *Paraxis* staining was abolished in the

caudal portion of these mutants, providing additional proof that contrary to *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ mutants lack somitic tissue. These results suggest that despite forming ectopic neural tissue, paraxial mesoderm fate is unaffected in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

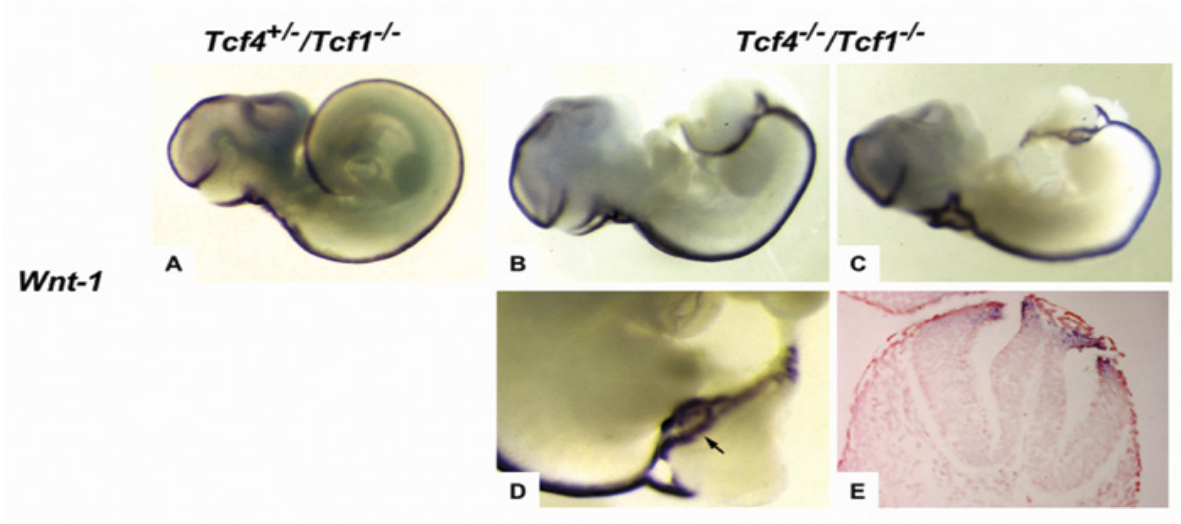

Figure 2. Duplications of the neural tube in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.
Whole-mount *in situ* hybridizations of E10.5 littermates using a *Wnt1* probe as a marker for neural tube. (A) *Tcf4*$^{+/-}$/*Tcf1*$^{-/-}$ embryo; (B) right side, (C) left side and (D) close-up views of a representative *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryo. Lateral and close-up views reveal that the neural tube appears to split at the caudal end of mutant embryos. In some regions (see arrow in D and the corresponding section in E) complete duplications of the neural tube are observed. Note that *Wnt1* expression appears normal in the anterior half of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

Next, we assessed whether expression of early caudal markers was impaired. Several genes expressed in the primitive streak and presomitic mesoderm have been reported to play an essential role during patterning of the caudal end of the mouse embryo. For example, mutations in *T* (*Brachyury*) cause a kinked tail phenotype and maintenance of *T* expression was shown to depend on intact TCF/LEF binding sites in its promoter (189;190). However, *in situ* hybridization on both E8.5 and E9.5 embryos demonstrates that *T* expression is unaltered in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos (Figure 4A-D). These results contrast analysis of *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos where maintenance of *T* expression was abolished in the tail bud of E9.5 embryos. We also tested *Tbx6*, another T box-containing family member implicated in posterior patterning (191). As shown in Figure 4E and F, *Tbx6* transcripts were still detected, although the levels and range of expression was reduced compared to normal littermates. *Cdx1* expression was shown to be down-regulated as early as E8.5 in *Wnt3a* deficient embryos, and in the fetal gut of *Tcf4*$^{-/-}$ mice (192;193). In spite of these observations, Figure 4G, and H show that *Cdx1* is not dependant on *Tcf1/Tcf4*, at least during gastrulation. Finally, we tested *Wnt5a* because of its essential role in expansion of the tailbud, but as with other markers tested (including *Cdx2, Lef, Evx1 and Wnt3a,* data not shown) no changes in expression were observed. Altogether these results provide further evidence that the patterning defects in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos cannot be directly attributed to loss of mesodermal precursors.

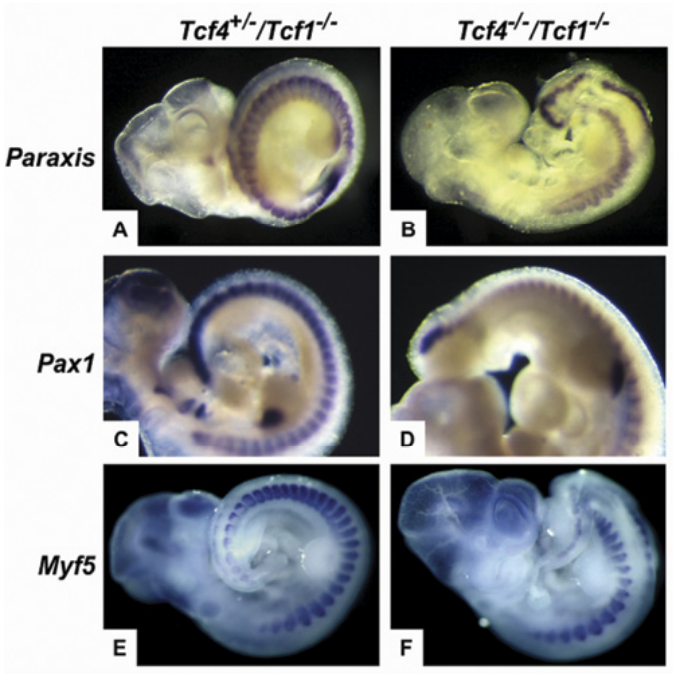

Figure 3. Paraxial mesoderm is intact in *Tcf4*$^{-/-}$ */Tcf1*$^{-/-}$ embryos.
Whole-mount *in situ* hybridizations of E10.5 littermates using markers for paraxial mesoderm. (A, C and E) *Tcf4*$^{+/-}$*/Tcf1*$^{-/-}$ embryos and (B, D and F) *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos. Panels A and B show stainings for *Paraxis,* Panels C and D show *Pax1* stainings. Panels E and F show expression of *Myf5*. All three somitic markers confirm the presence of paraxial mesoderm throughout the axis of *Tcf4*$^{-/-}$ */Tcf1*$^{-/-}$ mutants.

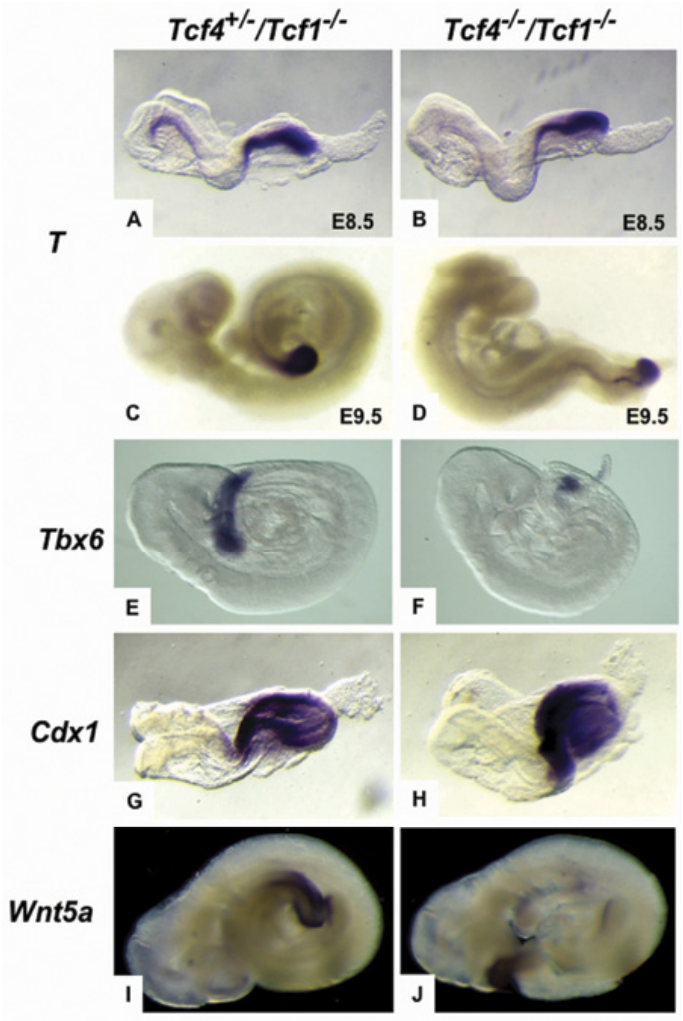

Figure 4. Expression of early posterior markers is unaffected in *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos.
Whole-mount *in situ* hybridizations of E8.5-9.5 littermates using various posterior markers (see above). (A, C, E, G, I) *Tcf4*$^{+/-}$*/Tcf1*$^{-/-}$ embryos and (B, D, F, H, J) *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos. Panels (A,B) and (C,D) represent E8.5 and E9.5 embryos respectively and show that initiation and maintenance of *T* expression is unchanged in *Tcf4*$^{-/-}$ */Tcf1*$^{-/-}$ embryos.

Expression of *Tcf4, Tcf1* and *Lef* in the primitive gut.

To gain further insight into the underlying causes of the *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ phenotype we reexamined the expression of *Tcf/Lef* family members. Previous analysis has shown overlapping expression of *Tcf1* and *Lef* in the forelimbs and primitive streak region of gastrulating embryos (61;194). Somewhat surprisingly, in light of the caudal truncations observed in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, *Tcf4* transcripts were mainly detected in rostral structures, such as di- and mesencephalon and pharyngeal arches (61;195). To address this discrepancy we performed *in situ* hybridization on sections of E8.5 and E10.5 wild-type embryos focusing our attention on posterior structures. Sections through the primitive streak showed that the overall expression pattern of *Tcf/Lef* genes was only partially overlapping. At E8.5 *Tcf4* was specifically detected in the hindgut. *Tcf1* was expressed in the primitive ectoderm, presomitic mesoderm and hindgut. *Lef* was restricted to the presomitic mesoderm (Figure 5A-C). At later stages, we could distinguish *Tcf4* expression in the neural tube and hindgut. Comparable sections showed that *Tcf1* expression was maintained in all three germ layers, including the neural tube, presomitic mesoderm and hindgut. Importantly, *Lef* was excluded from the primitive gut and was expressed in mesodermal structures (Figure 5D-F). The tissue specificities of *Tcf/Lef* genes provided a rationale to explain the disparities between the phenotypes of the compound null mutants. The overlapping expression of *Tcf1* and *Lef* in the presomitic mesoderm, coupled with the exclusion of *Tcf4* in this compartment, may explain why *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, but not *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, lose caudal somites. By analogy, we reasoned that the primary anomaly in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos may be impaired hindgut development. Based on these observations we decided to analyze endodermal fate in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

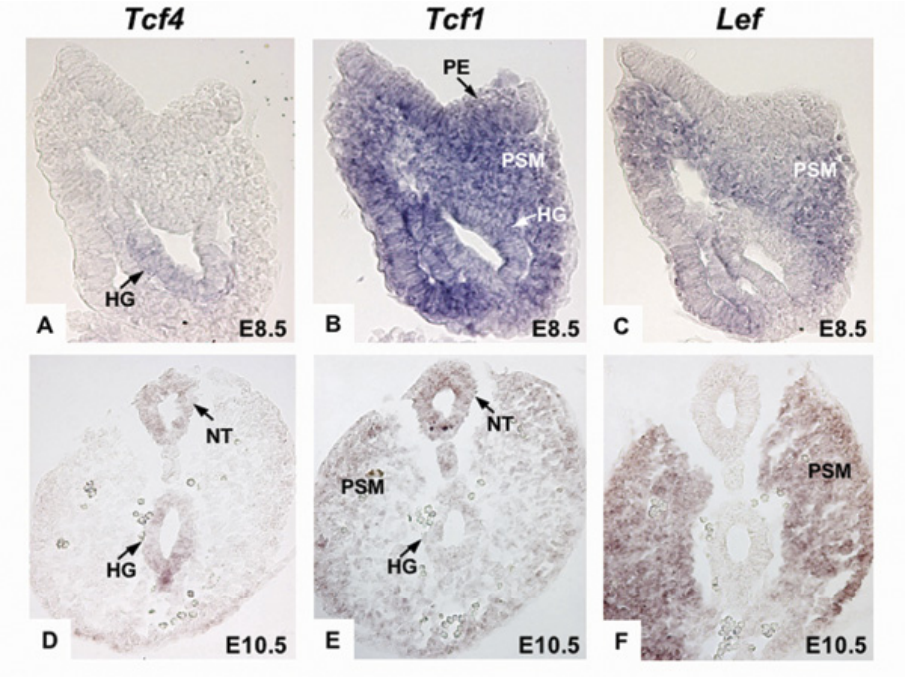

Figure 5. Comparison of *Tcf4, Tcf1* and *Lef* expression.
In situ hybridization on sections of E8.5 and E10.5 wild-type embryos were performed using specific probes recognizing *Tcf4* (A, D), *Tcf1* (B, E) and *Lef* (C, F). Top panels (A-C) depict sections through the primitive streak region of E8.5 embryos, while bottom panels (D-E) show sections through the tail bud of E10.5 embryos. Various structures are identified as follows: (PE) primitive ectoderm, (NT) neural tube, (PSM) presomitic mesoderm and (HG) hindgut. *Tcf4* was specifically expressed in the hindgut at E8.5 and at E10.5 in the neural tube and hindgut. *Tcf1* was found in all three germ layers at both E8.5 and E10.5. *Lef* was detected in the presomitic mesoderm in both stages examined and was absent from the primitive gut.

Caudal endoderm is impaired in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ but not *Lef*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

To follow gut development we analysed the expression of three endodermal markers *Sox17, Foxa1* and *Shh* in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ and *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos. At E8.5 endodermal tube formation is initiated by folding of the endodermal lining at the anterior and posterior ends creating anterior and caudal intestinal portals (AIP and CIP) [for reviews see (52;53)]. At this stage, *Sox17* and *Foxa1* are normally expressed along the entire endodermal lining in wild-type embryos (Figure 6A and D). In *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, however, the caudal endoderm showed an absence of both *Sox17* and *Foxa1* staining (Figure 6B and E, see arrows). Conversely, in *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos expression of both markers is uninterrupted (Figure 6C and F). At E9.5, the AIP and CIP join to close the endodermal tube resulting in formation of a proper foregut, midgut and hindgut. During this stage *Shh* expression is detected throughout the newly formed endodermal tube and clearly distinguishes the hindgut from the notochord at the posterior end (Figure 6G-I). In *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, only notochord staining is observed, indicating a

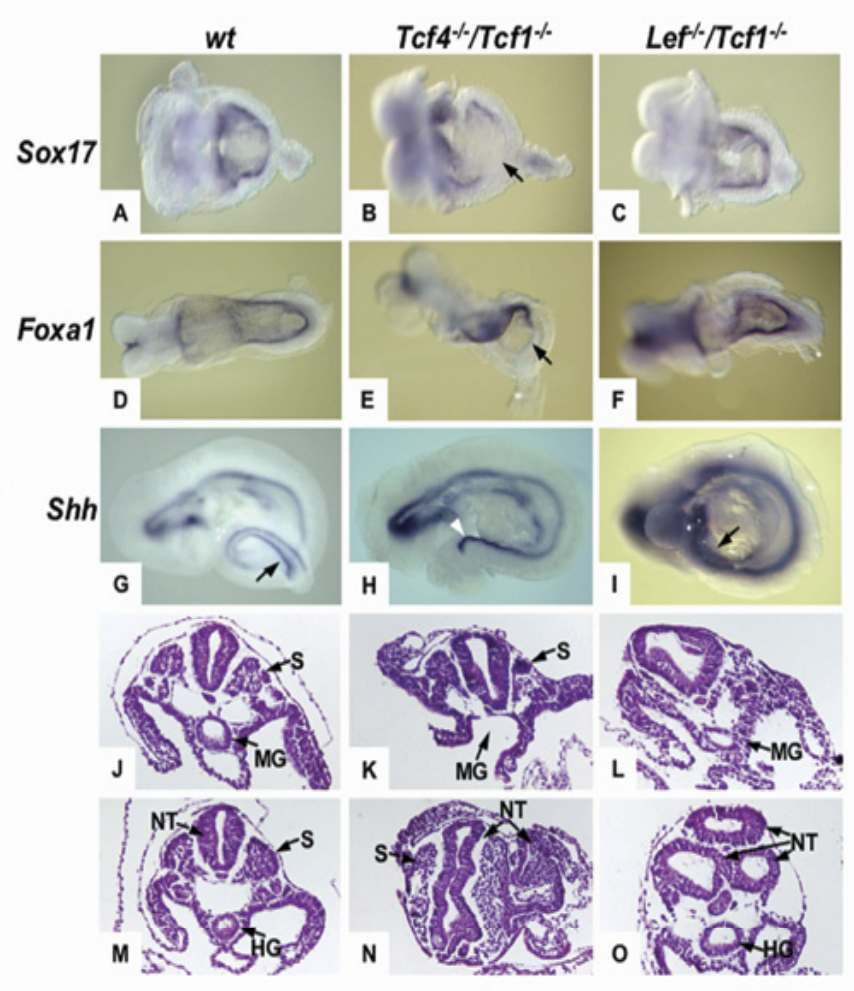

Figure 6. Impaired expansion of caudal endoderm in *Tcf4*$^{-/-}$ /*Tcf1*$^{-/-}$ embryos.
Whole-mount *in situ* hybridizations of E8.5-9.5 littermates using endodermal markers and histology of E9.5 normal and mutant embryos. (A,D,G, J, M) wild-type embryos and (B,E,H, K, N) *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos and (C,F,I, L, O) *Lef* $^{-/-}$ /*Tcf1*$^{-/-}$ embryos. The arrows in B and E point to the loss of *Sox17* and *Foxa1* staining in the caudal endoderm of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$, while staining is normal in *Lef* $^{-/-}$ /*Tcf1*$^{-/-}$ embryos. Arrows in G and I point to *Shh* expression in the hindgut of wild-type and *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos. The notochord in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$embryos appears exposed due the absence of an underlying gut tube (arrowhead in H). Panels J-L and M-O represent hematoxylin and eosin stainings of sections through the midgut region and hindgut respectively. Various structures are identified as follows: (S) somites, (NT) neural tube), (MG) midgut and (HG) hindgut. Note in panel K that *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos maintain somites but the primitive gut tube has not closed. Similar sections in *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos show a properly formed gut tube and the absence of somites (panel L). The caudal sections (M-O) also reveal the presence of ectopic neural tissue in both *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ and *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos.

complete lack of the hindgut. As a result of the loss of underlying gut, the notochord appears exposed at the tail end of these embryos (Figure 6H, see arrowhead). *Shh* expression in *Lef*$^{-/-}$*/Tcf1*$^{-/-}$ embryos was maintained in the hindgut (Figure 6I, see arrow), implying once again that the observed effects in *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ are specific. To confirm these data we prepared hematoxylin/eosin sections of wild-type, *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$, as well as *Lef* $^{-/-}$*/Tcf1*$^{-/-}$ embryos. Sections through the midgut region (just posterior to the stomach) of *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos revealed that the endodermal tube had not closed (Figure 6K). More caudal sections showed ectopic neural tissue and the absence of any hindgut (Figure 6N). Equivalent regions in *Lef*$^{-/-}$*/Tcf1*$^{-/-}$ embryos demonstrated that the gut tube had formed despite the loss of somites and formation of multiple neural tubes (Figure 6L and O). Altogether these results implied that specification and expansion of the hindgut is aberrant in *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos.

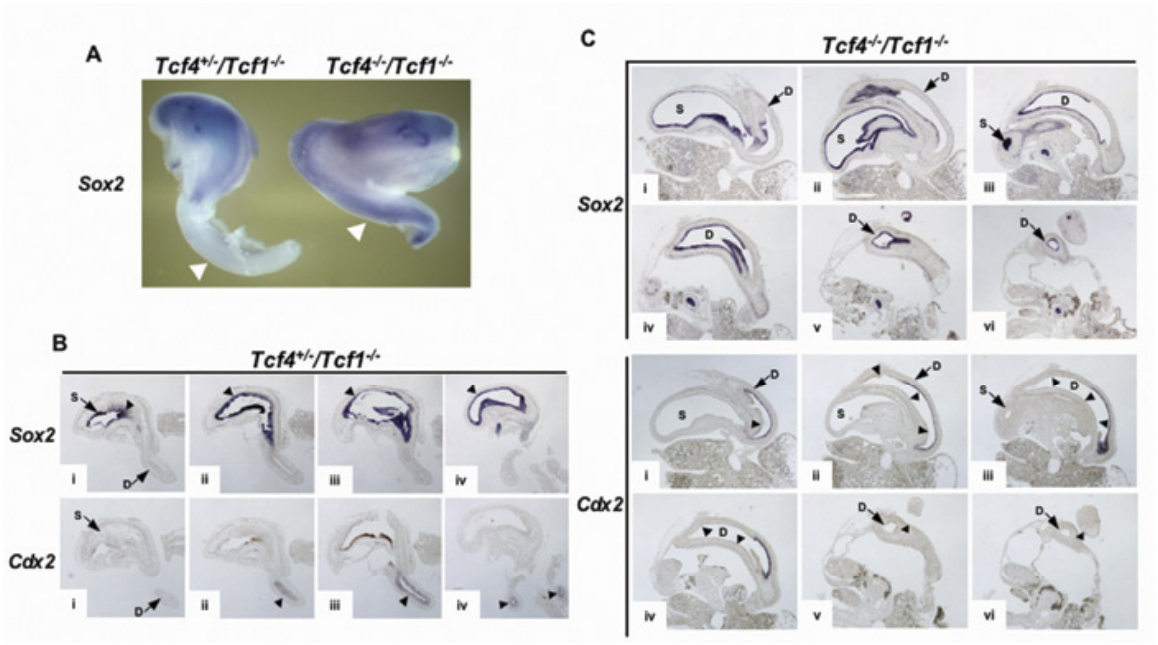

Figure 7. Anteriorization of the gastro-intestinal tract in *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos.
(A) Whole-mount *in situ* hybridizations of dissected anterior gastro-intestinal tracts of E14.5 *Tcf4*$^{+/-}$*/Tcf1*$^{-/-}$ (left) and *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ (right) embryos using a stomach marker, *Sox2*. The gastro-intestinal tract of the normal embryo was severed to remove the intestinal tube. The truncated gastro-intestinal tract of *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos is shown in its entirety. As shown by arrowheads, *Sox2* expression is confined to the stomach in normal littermates, while ectopic expression in the duodenum is apparent in mutants. (B) *In situ* hybridizations on consecutive sections (i-iv) of a single E13.5 *Tcf4*$^{+/-}$*/Tcf1*$^{-/-}$ gastro-duodenal preparation. Top and bottom rows of panels were stained for *Sox2* (stomach) and *Cdx2* (intestine) respectively. Top and bottom sections i-iv represent equivalent regions and should be compared with each other. Arrowheads indicate regions of positive staining for both probes (C) *In situ* hybridizations on consecutive sections (i-vi) of a single E13.5 *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ gastro-duodenal preparation. Top and bottom sets of panels were stained for *Sox2* and *Cdx2* respectively. Sections i-vi represent equivalent regions and should be compared with each other. Various structures are identified as follows (S) stomach, (D) duodenum. Arrowheads in panels stained for *Cdx2* represent regions of the duodenum which are devoid of *Cdx2* expression but show high expression of *Sox2*. Note how the duodenum in mutants appears dilated compared to normal littermates (B). In addition, *Cdx2* transcripts are confined to a small portion of the duodenum, the remaining tissue expresses *Sox2*. Altogether these data provide evidence for the occurrence of an anterior transformation in the gastro-intestinal tract of *Tcf4*$^{-/-}$*/Tcf1*$^{-/-}$ embryos.

Anterior transformation in the gastro-intestinal tract of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos.

Given the specific defects in the formation of caudal endoderm in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos we also were interested in examining later patterning events of the gastro-intestinal tract. As expected serial transverse sections of E14.5 embryos showed that the intestine of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ mutants was severely truncated and ended blindly {Supplementary Figure 1 (see http://www.nature.com/ emboj/journal/v23/ n8/extref/7600191s1.pdf, bottom panels m-o}. Closer inspection of mutant embryos revealed profound patterning defects in the stomach/duodenum region. Normally, the stomach is restricted by the oesophageal junction at the proximal end and by the duodenal junction at the distal end {Supplementary Figure 1 (see http://www.nature.com/ emboj/journal/v23/n8/extref/7600191s1.pdf) top panels a-e}. While the boundary between the oesophagus and stomach was normal in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, the duodenal opening was not clearly defined. In fact, serial sections suggested that the duodenum was dilated, giving the impression that the stomach had duplicated at the distal end {Supplementary Figure 1 http://www.nature.com/ emboj/journal/v23/n8/extref/ 7600191s1.pdf, bottom panels}. To better visualize this we dissected out the stomach and intestine from E14.5 embryos in order to verify the expression of the epithelial stomach marker *Sox2* (196). In *Tcf4*$^{+/-}$/*Tcf1*$^{-/-}$ littermates *Sox2* expression was restricted to the stomach with no transcripts detected beyond the duodenal boundary. In *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, *Sox2* expression was present throughout the stomach and truncated intestinal tract. These results implied that the *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ gastro-intestinal tract was indeed anteriorized. To confirm this we performed *in situ* hybridisations on consecutive sections of normal and mutant stomach/duodenum preparations in order to verify the expression of the intestinal marker, *Cdx2,* alongside *Sox2*. As depicted in Figure 7, *Sox2* and *Cdx2* are confined to the stomach and duodenum respectively in normal littermates (see Figure Legends for details). In mutant embryos, comparable sections showed that the duodenum only partially expressed *Cdx2*. Moreover, regions of the duodenum devoid of *Cdx2* (see black arrowheads, comparing top and bottom sets of panels i-vi) abundantly expressed *Sox2*. Thus, the duodenum was apparently transformed into stomach, effectively leaving *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos with little or no intestine.

Discussion

In this report we show that deletion of *Tcf1* and *Tcf4* leads to an absence of caudal structures in the mouse embryo. These embryos displayed duplications of the neural tube and unlike other null mutants affecting Wnt signalling, *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos retain paraxial mesoderm. By examining the expression of endodermal markers we found an early defect in the development of the hindgut and later patterning anomalies consistent with homeotic transformations of the gastro-intestinal tract. The specific defects in caudal endoderm and later axial truncations are reminiscent of recent experiments with chick embryo explants, where surgical removal of caudal endoderm leads to blunted tail development (197). Likewise, the impaired gut development in *Sox17*$^{-/-}$ embryos is accompanied by disorganized posterior trunk development which can partially be rescued in chimeras (58;198). Together these findings suggest that posterior endoderm may produce signals required for proper development not only of the hindgut but also adjacent structures. Therefore in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, the basis for the severe posterior truncations may be the result of arrested gut development. In light of this, the disorganized aspect of the paraxial mesoderm in some embryos may be attributed to the hindgut defects. Further analysis will be required to test this hypothesis.

Tcf/Lef genes regulate distinct patterning events

The comparison between *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ and *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos has allowed us to uncover distinct roles for *Tcf/Lef* family members during A-P patterning. Previous work and our own analysis indicate that *Lef* and *Tcf1* are necessary for early specification of paraxial mesodermal. *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ embryos phenocopy null mutations in *Wnt3a* and also the putative target gene *Tbx6* (191). The primary defect in these mutants appears to result from the formation of ectopic neural tissue at the expense of paraxial mesoderm. One model that has emerged to explain this phenomenon suggests that Wnt signals (namely Wnt3a–LEF/TCF1) instruct epiblast cells to adopt a mesodermal fate. When this signal is disrupted, involuting cells from the primitive streak fail to differentiate into mesoderm, and possibly by default, adopt a neuronal differentiation program, ultimately contributing to the formation of ectopic neural tissue where normally somites are formed.

In *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos, expression of posterior paraxial mesoderm markers is maintained, suggesting that *Tcf4* is not required for specifying this tissue type. Rather *Tcf4* in concert with *Tcf1* are necessary for the development of caudal endoderm. By analogy to the above model our results could also imply that during germ layer formation, *Tcf4* and *Tcf1* regulate the formation and/or expansion of caudal endoderm at the expense of neural tissue. Thus the neural tube duplications in these embryos may result from unspecified epiblast cells forming ectopic neural tubes as described in *Wnt3a*$^{-/-}$ and *Lef* $^{-/-}$/*Tcf1*$^{-/-}$ mutants. Alternatively, the duplications in *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos may reflect later intrinsic patterning defects caused by loss of *Tcf4* and *Tcf1* expression in the neural tube.

Wnt signals, therefore, pattern specific caudal structures through the differential usage or tissue distribution of *Tcf4*, *Tcf1* and *Lef*. The absence of any defect in either paraxial mesoderm or caudal endoderm development in the single knockouts, however, argues that TCF/LEF factors are functionally redundant at the biochemical level. In other words, LEF/TCF1 in the paraxial mesoderm and TCF4/TCF1 in the caudal endoderm most likely regulate overlapping sets of target genes. However, these results do not exclude the possibility that within other cell types, co-expressed TCF/LEF factors control unique genetic programs. In favour of this notion, promoter studies have demonstrated that unique domains present within individual TCF/LEF proteins allow for the regulation of specific target genes (199;200). Moreover, forced expression of mutant version of *Tcf3* and *Lef* in different model systems, such as *Xenopus* embryos and skin stem cells, results in distinct developmental outcomes (201;202). Thus, it appears that the exact contribution of individual TCF/LEF factors in response to Wnt signals depends on the cellular and developmental context.

Both early and late stages of gastro-intestinal development are controlled by Wnt signalling

The specific defects in the fetal gut development of *Tcf4*$^{-/-}$/*Tcf1*$^{-/-}$ embryos are consistent with our previously described single *Tcf4* knockout (66). The phenotype of these mice first becomes evident during villus formation in the small intestine at around E16.5 At this time point, proliferating epithelial cells normally detected in the inter-villus regions are lost and as a result *Tcf4*$^{-/-}$ mice die perinatally. Given the importance of *Tcf4* in maintaining epithelial stem cells in the small intestine, it is intriguing to speculate that *Tcf4* may play an equivalent role in the primitive gut. A recent study in the chick provides additional evidence that *Tcf/Lef* factors are essential for gut development (203). However, in this model expression profile analysis and loss of function studies predict a restricted role for *Tcf4* and *Lef* in the development of the gizzard, the duodenum and ceca.

The transformations of the gastro-intestinal tract highlight an unexpected role for *Tcf4* and *Tcf1* in later patterning of the gut tube. From E9.5-E14.5, once the endodermal tube has formed, specialized structures such as lungs, stomach, small intestine, colon etc. develop along the A-P axis. During this patterning phase, both *Tcf4* and *Tcf1* are expressed in foregut derivatives such as the stomach (data not shown). *Tcf4* is strongly expressed in the stomach epithelium. *Tcf1* expression appears much less abundant and transiently, with early expression in the stomach mesenchyme and later in the epithelium. Because removal of *Tcf4* and *Tcf1* leads to ectopic stomach tissue, this may reflect a role for both factors in restricting growth of the stomach while at the same time promoting growth of more caudal endodermal structures (ie. the midgut and hindgut).

Besides regulating the expansion and patterning of the gastro-intestinal tract, a number of other functional studies implicate canonical Wnt signalling in other aspects of gut development. For example, the initial formation of definitive endoderm is severely impaired in conditional *β-catenin* mutant embryos; and as a result these embryos develop ectopic cardiac tissue (55). As a self-renewing tissue, the adult intestine also requires active Wnt

signalling to maintain proliferation of epithelial progenitors. This was confirmed recently by two independent studies which showed that forced expression of the Wnt antagonist, *Dkk1*, blocks proliferation in the intestinal mucosa (67;68). On the other hand, several lines of evidence have shown that over-stimulation of the Wnt pathway, through activating mutations in components such as *β-catenin* and *APC*, promotes tumorigenesis (4;87). Altogether therefore these data imply that Wnt signalling is absolutely required for the formation, growth, patterning and homeostasis of the gut endoderm.

In conclusion, the results presented here establish a novel role for Wnt signalling in early gut morphogenesis and underscore the importance of the *Tcf/Lef* genes in driving early patterning events during mouse development. Given the abundance of Wnts (19 described genes) and the probability that many have overlapping functions, future analysis of various compound *Tcf/Lef* null mutants should provide valuable models to dissect the role of canonical Wnt signalling in many other developmental processes.

Experimental Procedures

Mice

*Tcf*4, *Tcf1* and *Lef* deficient mice were described elsewhere (66;185;187). Genotyping of all embryos was performed by PCR of genomic DNA isolated from extraembryonic tissue. Detailed protocols (including primer sequences) for genotyping will be provided upon request.

Histology and in situ hybridization

Embryos were dissected in PBS, fixed overnight in 4% paraformaldehyde and kept for long term storage in 100% methanol. For whole-mount *in situ* hybridization, embryos were rehydrated, digested in proteinase K, post-fixed, and hybridized overnight at 70°C with various probes in 5X SSC (pH 4.5), 50% Formamide, 2% Blocking Powder (Roche), 5mM EDTA, 50µg/ml yeast tRNA, 0.1% Tween 20, 0.5%CHAPS and 50µg/ml Heparin. Embryos were washed as follows: 2X SSC for 4X 10minutes at 70°C, 2X SSC/0.1% CHAPS for 2X 30 minutes at 70°C, 100µg/ml RNAse A/2X SSC/0.1% CHAPS for 30 minutes at 37°C, and 100mM maleic acid/150mM NaCl for 2X 30 minutes at 70°C. Embryos were washed in TBST, blocked for 2 hours in TBST containing 0.5% Blocking Powder and 1% sheep serum. Next embryos were incubated in blocking solution overnight at 4°C with preabsorbed alkaline phosphatase-conjugated anti-digoxigenin (1/2000 dilution) (Roche). Embryos were washed several times in TBST and color reaction was performed with BM Purple AP substrate (Roche). For *Wnt1* stainings embryos were dehydrated, embedded in paraffin, sectioned and counterstained with 1.0% Neutral Red. For *in situ* hybridization on sections embryos were embedded in paraffin, sectioned at 10µM and processed for hybridization as above with the following modifications. Both CHAPS and Tween 20 were omitted from the hybridization buffer and post hybridization washes were performed as follows: 2X SSC for 5 minutes, 2X

SSC/50% Formamide for 3X 30 minutes at 65°C. Sections for histology studies were stained with hematoxylin and eosin.

Probes

The following probes were used for *in situ* hybridization studies: *Pax1* (IMAGE clone #1327502), *T* (204), *Wnt5a* (205), *Cdx1* (206), *Lef* (194), *Foxa1* (207), *Sox17* (58), *Sox2* (IMAGE clone # 5707193) *Tcf1* (IMAGE clone #4016305) *Tcf4* (IMAGE clone #4952976)

CHAPTER 3

Expression pattern of Wnt signaling components in the adult intestine

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Expression pattern of Wnt signaling components in the adult intestine

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Abstract

Background & Aims: In the intestine the canonical Wnt signaling cascade plays a crucial role in driving 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, Fzs, LRPs, Wnt antagonists and TCFs in the murine small intestine, colon and adenomas. Methods: Embryonic, postnatal and adult intestinal samples were subjected to *in situ* hybridisation using specific RNA probes for the various genes tested. Results: Our analysis revealed high expression of several signaling components (including *Wnt-3, Wnt-6, Wnt-9b, Fz-4, Fz-6, Fz-7, LRP-5* and *sFRP-5*) in crypt epithelial cells. We also detected *Wnt-2b, Wnt-4, Wnt-5a, Wnt-5b, Fz-4 and Fz-6* in differentiated epithelial and/or mesenchymal cells of the small intestine and colon. Finally, several factors (*Fz-4, TCF-1, LEF, Dkk-2, Dkk-3 and WIF*) displayed differential expression in normal versus neoplastic tissue. Conclusion: 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, as well as non-canonical Wnt signals in the intestine.

Introduction

The intestine harbors a variety of cell types, 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 (104;208). One such example includes the Wnt family of secreted growth factors (13;209;210). Wnts are evolutionarily conserved, cysteine-rich glycoproteins, capable of signaling in both paracrine and autocrine fashion. Wnts induce a wide range of biological effects by binding to the seven-span transmembrane protein, Frizzled (Fz) (20) and the single-span LDL receptor-related protein, LRP (21-23). 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 (209). Subsequently, free β-catenin shuttles to the nucleus where it associates with DNA binding factors of the TCF (T-cell factor) family in order to activate transcription of target genes (39;40). More recent evidence has shown that Wnts may also stimulate cellular responses, independently of β-catenin and TCF (25;211). Examples of these so-called non-canonical pathways involve either the intracellular release of calcium ions and activation of Ca^{++} dependent kinases or morphogenic changes dependent on RhoA and Jun kinase stimulation, also termed the planar cell polarity pathway.

Genetic studies have demonstrated 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 APC and β-catenin (212-214). 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 (73;215-217). Conversely, blockage of canonical Wnt signals in the intestine, either through deletion of *TCF-4* or overexpression of the Wnt antagonist, Dkk-1, results in arrested epithelial cell proliferation (66-68). Another function ascribed to Wnt signaling in epithelial cells was recently uncovered through DNA micro-array analysis. EphB2 and B3 are TCF-4-responsive genes, which regulate upward movement of epithelial cells and the positioning of Paneth cells within the crypt-villus axis (86). Together these data provide clear evidence that proliferation, as well as sorting of crypt epithelial cells depends on the correct dosage of canonical Wnt signals.

Despite the functional evidence described above, 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 (203;218;219). In the adult intestine Wnt expression is maintained (220) 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, as well as TCF family members in the adult intestine by *in situ* hybridization (ISH).

Results

Expression of Wnt genes in the adult intestine.

In order 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), LRPs (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

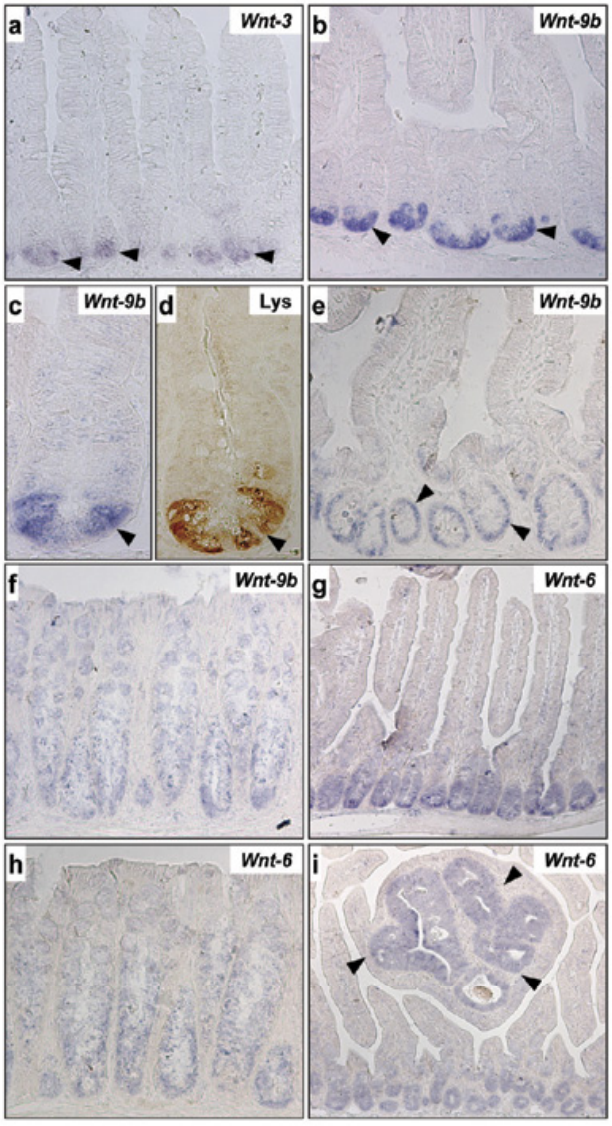

Figure 1. ISH analysis of Wnts expressed in intestinal epithelial cells.

Panels (a) shows *Wnt-3* expressed in Paneth cells (arrowheads). Panel (b and c) show Wnt-9b strongly and predominately expressed in Paneth cells in the distal portion of the intestine (ileum). Panel (d) represents a consecutive 4µm section of the same intestinal crypt as shown in panel (c) and was immunostained with an antibody recognizing the Paneth cell marker, lysozyme. Panel (e) represents a section of the duodenum and shows *Wnt-9b* expression in Paneth cells and crypt progenitor cells. Panel (f) shows *Wnt-9b* expressed throughout the colonic epithelium. Panels (g-i) show *Wnt-6* in crypt epithelial cells of the small intestine and colon, as well as in adenomas respectively.

Wnts were readily detected in the intestine {results are recapitulated in Figure 6 and Supplementary Table I (http://www.niob.knaw.nl/ researchpages/clevers/files)}. As shown in Figures 1 and 2, 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 1a). 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 1b). To confirm the identity of these cells, we performed immunostainings on consecutive 4μm sections for the classical Paneth cell marker, lysozyme. As shown on corresponding sections (Figure 1c and d) of the same intestinal crypt, *Wnt-9b*-expressing cells also produced lysozyme and thus represent Paneth cells. Interestingly, in more proximal areas of the small intestine *Wnt-9b* was also detected in epithelial progenitor cells above the Paneth cell compartment (Figure 1e). Contrary to the small intestine, in the colon *Wnt-9b* was localized throughout the colonic epithelium (Figure 1f). 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 (221). Finally, we found *Wnt-6* expressed throughout the crypts of the small intestine and colon (Figure 1g and h). Similarly, *Wnt-6* was strongly expressed in adenomas (Figure 1i).

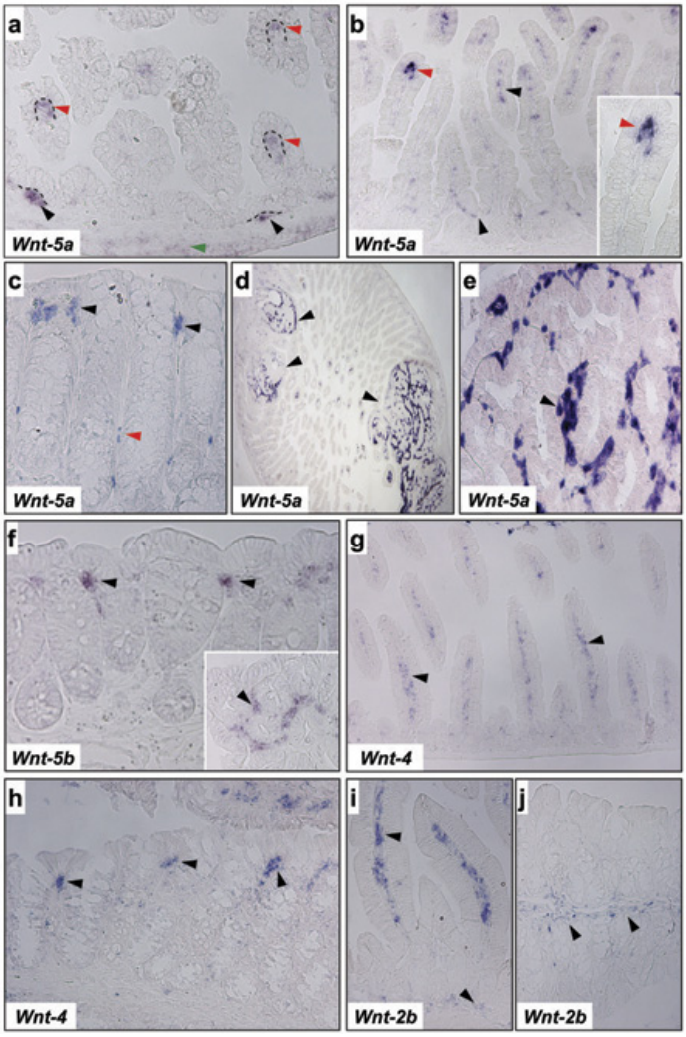

Figure 2. ISH analysis of Wnts expressed in the mesenchyme of the small intestine and large intestine.
Panel (a) shows at E18.5 *Wnt-5a* accumulation in the mesenchyme of the villi tips (red arrowheads), in mesenchymal cells adjacent to proliferative epithelial cells (black arrowheads), and in the smooth muscle layer (green arrowhead). The border between the epithelium and the mesenchyme is indicated with dashed lines. In the mature intestine (panel b), *Wnt-5a* is strongly expressed in mesenchymal cells at the tips of villi (red arrowheads and inset), while levels are reduced along the villi towards the crypt-villus border (black arrowheads). Panel (d) and a higher magnification in panel e show *Wnt-5a* upregulation in stromal cells of intestinal polyps (black arrowheads). Panels (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 panel (f) shows cross-section of colonic crypts with *Wnt-5b* staining in surrounding mesenchyme. Panel (g) shows uniform *Wnt-4* expression throughout the villi. Panels (i) and (j) show *Wnt-2b* in mesenchymal cells of the small intestine and endothelial or smooth muscle cells of the colon, respectively.

Several Wnts were also found in specific compartments of the mesenchyme. In the fetal gut, *Wnt-5a* showed high expression at the tips of growing villi and in few discrete cells beneath the proliferative epithelium (Figure 2a). 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 2b). In the colon, *Wnt-5a* and the closely related *Wnt-5b* were restricted to the mesenchyme beneath the surface epithelium (Figure 2c and f). Lastly, *Wnt-5a,* and to a lesser extent *Wnt-5b*, were both upregulated in stromal cells of APC^{min} polyps (Figure 2d, 2e and data not shown). Unlike *Wnt-5a*, *Wnt-4* was uniformly expressed along the villus mesenchyme and absent from adenomas (Figure 2g and data not shown). However, in the colon *Wnt-4* expression closely resembled *Wnt-5a* and *5b* stainings (Figure 2h). Finally, *Wnt-2b* was strongly expressed in the mesenchymal layer of the villi and more weakly in the crypts of the small intestine (Figure 2i). In the colon, *Wnt-2b* transcripts were less abundant and appeared to be markers of endothelial or smooth muscle cells (Figure 2j).

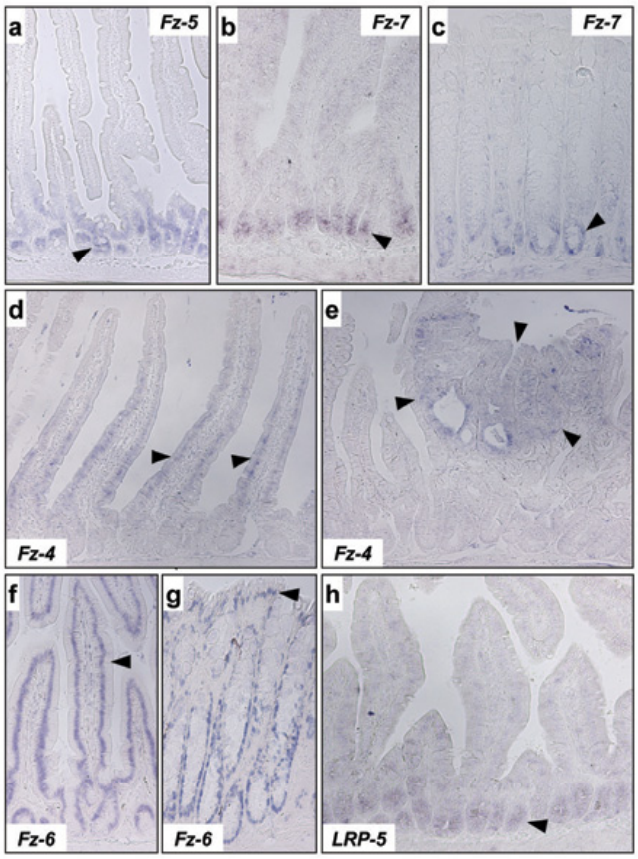

Figure 3. ISH analysis of Wnt receptors in the intestine and colon.
Panel (a) shows *Fz-5* in the epithelium of the crypts and crypt-villus border. Panel (b) and (c) show *Fz-7* in the lower portions of adult intestinal and colonic crypts, respectively. Panel (d) shows *Fz-4* in differentiated epithelial cells of the villi. Note that expression of *Fz-4* is strongest in the lower half of the villi. Panel (e) shows *Fz-4* upregulated in intestinal polyps. Panel (f) and (g) show *Fz-6* throughout the epithelium in both small and large intestine, respectively. Panel (h) shows *LRP-5* restricted to proliferative epithelial cells.

Expression of Wnt receptors in the adult intestine

In order to identify putative Wnt-responsive cells, we next examined the expression of Frizzled related receptors 1-10. As indicated in Supplementary Table I (see http://www.niob.knaw.nl/researchpages/clevers/files) and Figure 3, several *Fz* genes were located in both the fetal and adult intestine. As shown in Figure 3a and in van Es et al. (57), 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 inter-villus

epithelial cells of neonates (P1) (data not shown); although at later stages, *Fz-7* was confined to the epithelium of the crypt bottom (Figure 3b and c). *Fz-4* transcripts were also widely distributed in neonatal guts (Supplementary Table I), while expression in adults was comparatively weaker and restricted to differentiated epithelial cells of the villi (Figure 3d). One notable exception to this was the high levels of *Fz-4* in adenomas, as shown in Figure 3e. Finally, *Fz-6* was uniformly expressed throughout the epithelium of the small and large intestine (Figures 3f and g). We also tested the expression of LRP co-receptors, *LRP-5* and *6*, given their absolute requirement in driving canonical Wnt signals. As expected we found both *LRP-5* and *6* expressed in proliferative epithelial cells of the crypts (Figure 3h and data not shown).

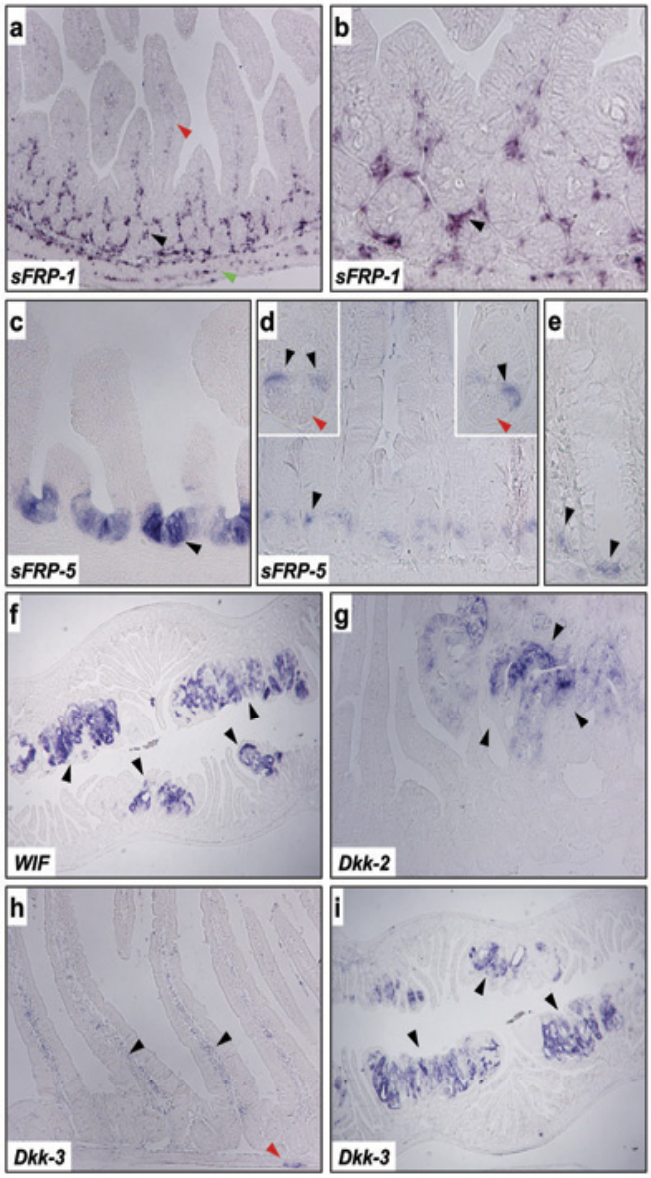

Figure 4. ISH analysis of secreted Wnt antagonists in the small intestine.
Panel (a) and the close-up in panel (b) show *sFRP-1* in mesenchymal cells surrounding the crypts. Additional "spotty" staining is also detected in the smooth muscle layer (green arrowhead), while expression in the villi is reduced (red arrowhead). Panel (c) shows *sFRP-5* expression in all proliferative epithelial cells of the fetal (E16.5) intestine. Panel (d) shows *sFRP-5* staining of single crypt epithelial cells in the adult small intestine. Insets show close-ups of two typical examples of *sFRP-5* expression in single cells (black arrowheads), just above the Paneth cells (red arrowheads). Panel (e) shows *sFRP-5* expression limited to the base of two consecutive colonic crypts. Panel (f) and (g) show *WIF* and *Dkk-2* respectively, expressed only in intestinal polyps of APC^{min} mice (black arrowheads), while normal epithelium is devoid of any expression. Panel (h) shows *Dkk-3* expressed in mesenchymal cells of the villi (black arrowhead) and plexi of the enteric nervous system (red arrowhead). Panel (i) shows *Dkk-3* strongly up-regulated in APC^{min} polyps.

Expression of secreted Wnt antagonists in the adult intestine

The functional counterparts of the Fz receptors include a family of secreted factors termed sFRPs (18;222). 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 five *sFRP* probes tested here, we only detected expression of *sFRP-1* and *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 4a, b and data not shown). *sFRP-5* was strongly expressed in epithelial progenitor cells at E16.5 (Figure 4c). 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 (Figure 4d and e) (64;223;224). 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 (http://www.niob.knaw.nl/researchpages/clevers/files)}. 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 (225-227). To test this we examined both *sFRP-5* and *Musashi-1* expression by in situ hybridization on consecutive sections. As shown in Supplementary Figure 2a and b, we found that *sFRP-5* displayed a more localized expression pattern than *Musashi-1*. Indeed, we observed the latter was expressed throughout the epithelial progenitor cell compartment. Lastly, it is worth noting that *sFRP-1* and *sFRP-5* were not detected in intestinal polyps (data not shown).

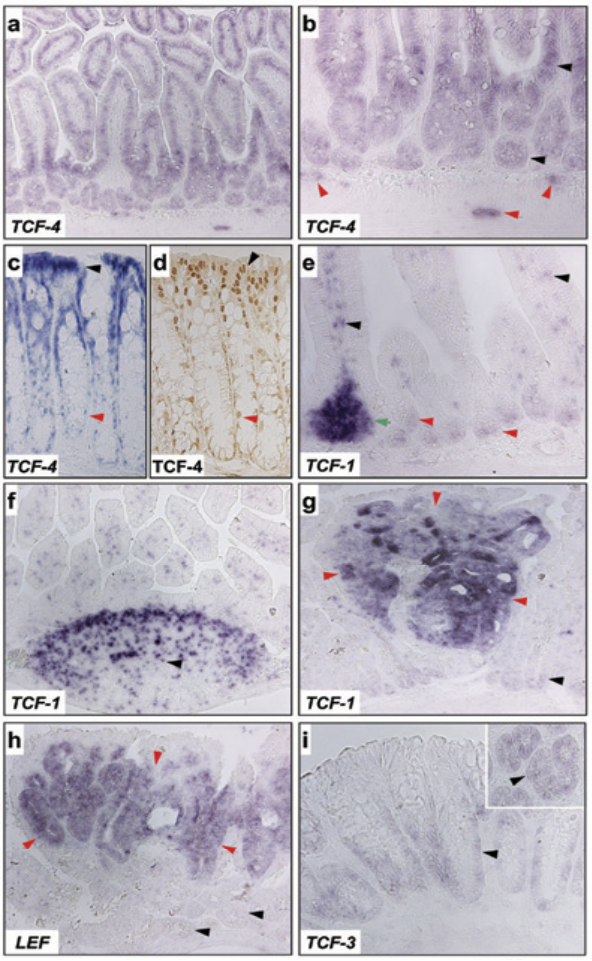

Figure 5. ISH analysis of TCFs in the small and large intestine.
Panel (a) and the higher magnification in panel (b) show *TCF-4* throughout the epithelium of the small intestine (black arrowheads). *TCF-4* also accumulates in the submucosal and myenteric ganglia of the enteric nervous system (red arrowheads). Panel (c) shows high expression of *TCF-4* in the upper half or non-cycling areas of the colonic crypts (black arrowheads), while expression is reduced in the lower half or proliferating cells (red arrowheads). Panel (d) shows a similar expression gradient with *TCF-4* antibodies. Panel e shows *TCF-1* in epithelial cells of the crypt bottom (red arrowheads), in gut-associated lymphoid tissue (GALT) (green arrowhead) and putative infiltrating intestinal lymphocytes (black arrowheads). Panel (f) shows high expression of *TCF-1* in Peyer's patches. Panel (g) shows upregulation of *TCF-1* in adenomas (red arrowheads) when compared to normal crypt epithelium (black arrowheads). Panel (h) shows ectopic expression of *LEF* in intestinal polyps (red arrowheads), whereas normal epithelium is devoid of *LEF* (black arrowheads). Panel (i) and inset show unique *TCF-3* expression in epithelial cells of colonic crypts.

Like sFRPs, other secreted factors can inhibit Wnt action by binding directly to Wnts as in the case of WIF and Cerberus (18;222) or by binding to LRP co-receptors, as with the Dkk class (24). In Figure 4f we found *WIF* uniquely expressed in adenomas, confirming a recent report (228). Similarly, a *Dkk-2* probe specifically stained adenomas (Figure 4g), whereas *Dkk-3* was weakly expressed in the villus mesenchyme, myenteric plexi, and upregulated in adenomas (Figure 4h and i). *Dkk-1, Dkk-4* and *Cerberus* were undetected in the intestinal tract (data not shown).

Expression of TCF family members in the intestine

Numerous studies have examined the function of TCFs in gut development and cancer (38). As shown in Figure 5a and b, a *TCF-4* probe revealed 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 non-proliferative cells was not anticipated based on the available genetic data (66), we wished to confirm the ISH results with TCF-4 antibody stainings. When comparing equivalent regions within the colon (see panels 5c and d), we once again found TCF-4 expressed predominately in non-cycling 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, implying that TCF-1 acts downstream of TCF-4/β-catenin and may play a role in cancerogenesis (145). In agreement with this, we found *TCF-1* expression strongly up-regulated in adenomas when compared to normal proliferative crypt cells (Figure 5g). However, we also observed *TCF-1* transcripts in infiltrating gut lymphocytes, gut-associated lymphoid tissue (GALT) and Peyer's patches (Figure 5e and f). This result is supported by previous findings showing a crucial role for TCF-1 in thymocyte development (187). As with *TCF-1,* we also detected *LEF* expression in intestinal polyps, although in normal epithelium, *LEF* transcripts were absent (Figure 5h). Similar findings were observed in human colorectal tumours, in which case *LEF* expression is directly regulated by TCF-4/β-catenin (146). Lastly, 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 (195). Likewise, our analysis revealed expression of *TCF-3* in proliferative compartment of the colon, although we were unable to detect *TCF-3* elsewhere (Figure 5i).

Discussion

In this paper we present an exhaustive overview of the expression pattern of Wnt signaling components in the murine intestine. As summarized in Figure 6 and Supplementary Table I (http://www.niob.knaw.nl/researchpages/clevers/files), 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.

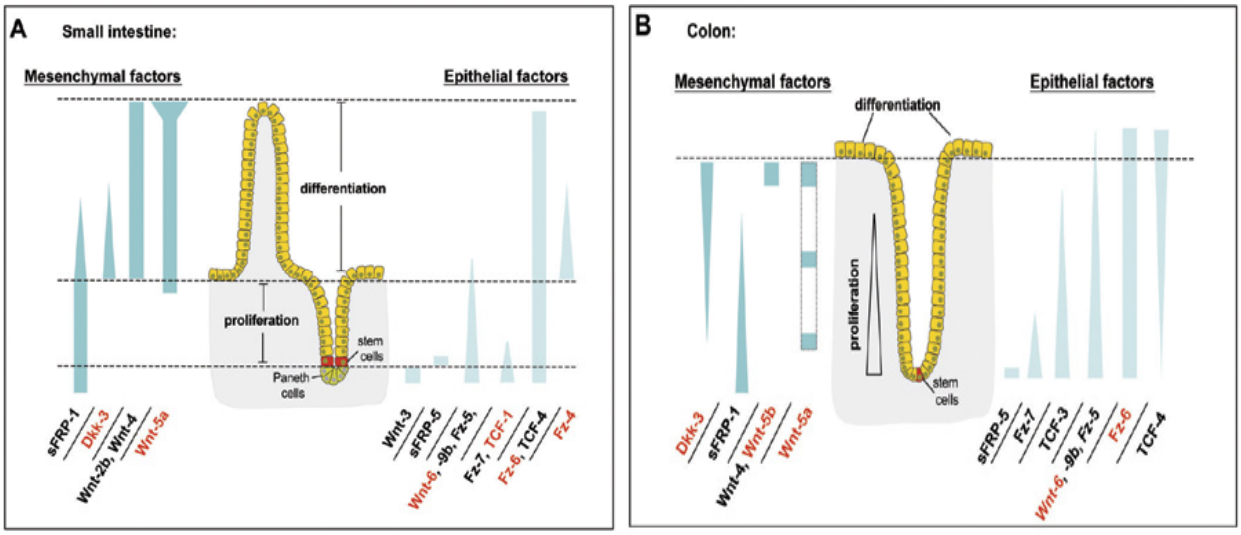

Figure 6. Summary of expression data.
Panels A and B show schematic representations of the small intestine and colon, respectively. The epithelium is shown in yellow while the mesenchymal layer is depicted in grey. Epithelial compartments associated with proliferation and differentiation are also shown. For simplicity only the expression pattern of Wnt signaling components which are most relevant to epithelial proliferation/differentiation are shown. Factors highlighted in red are also upregulated in adenomas. Other factors such as *WIF, Dkk-2 and LEF* are not expressed in the normal intestine but are ectopically expressed in adenomas.

Canonical and non-canonical Wnt signaling in the gut

In Figure 1 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 (86). EphB3 in combination with its ligand ephrin B1 force Paneth cells to occupy the very bottom of the crypts. Therefore, based on 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 maturation of Paneth cells. We and others have recently shown that expression of Paneth cell markers, such as anti-microbial peptides (i.e. cryptdins and defensins), is dependent on active Wnt/β-catenin signals (102;103).

Alternatively, Wnt-3 and 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 3).

Along the same lines, recent immunohistological studies and *in vitro* functional assays have identified Wnt-11 as another candidate implicated in promoting proliferation (229).

Contrary to the Wnts discussed above, our study has also identified candidate Wnts likely involved in inducing non-canonical 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 2). In these regions, epithelial and mesenchymal cells are devoid of nuclear β-catenin and TCF target genes are turned off, implying that the above Wnts do not activate β-catenin/Tcf in the intestine (230). Several lines of evidence suggest that non-canonical Wnt signals regulate a complex array of cellular responses including increased calcium flux, repression of TCF-mediated transcription and cytoskeletal rearrangements (25;211). Therefore, in the intestine Wnt-2b, Wnt-4, Wnt-5a, 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 (231). 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 non-canonical Wnts discussed above 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 3). Similarly in adenomas, Fz-4 and Wnt-5a are upregulated in epithelial and stromal cells, respectively. Considering that Fz-4 is known to physically interact with Wnt-5a (32), these factors may coordinate cross-talk between epithelial and mesenchymal cells. Finally, functional and biochemical evidence suggest that Fz-6 is an important mediator of planar cell polarity signals in mammalian epithelial cells (232) and can actively repress canonical Wnt signals (233).

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, which may fulfill this function. *sFRP-1*, for example, was localized to the crypt mesenchyme, co-expressed and adjacent to cells abundantly expressing Wnts (Figure 4). Consequently, sFRP-1 may function to modulate the effects of Wnt signaling in these regions. Indeed, a recent survey revealed that *sFRP-1* was frequently downregulated in colorectal cancers, implying a tumour suppressor role for this factor (151). Moreover, forced expression of sFRPs block proliferation of colorectal cell lines (234). Similarly, WIF, as well as Dkk-2 and Dkk-3 may also play a role in tumorigenesis based on their ectopic expression in adenomas (Figure 4). 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 has shown that Dkk-3 is unable to block Wnt signaling (235;236); whereas Dkk-2 can synergize with Fz

receptors to induce Wnt signaling responses (237;238). Finally, *sFRP-5* was strongly expressed in single epithelial cells of the bottom of crypts (Figure 4 and Supplementary Figure 2). Labeling experiments have shown that stems cells, the number of which may vary from 1-6 per crypt are located in equivalent positions just above the Paneth cells in the small intestine and at the crypt base in the colon (64;223;224). We are presently investigating whether *sFRP-5* positive cells represent *bonefide* stem cells by gene deletion in the mouse germ line.

Diverse functions for TCFs in the gut

In this manuscript 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 tumour suppressor regulating proliferation of epithelial cells in the intestine (145). 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 5), 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 (239). Accordingly TCF-4, in differentiated, β-catenin inactive cells, may be required to suppress target genes associated with proliferation. On the other hand, 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 (179), 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 non-canonical pathways. In turn, this study reveals a broad role for Wnt signaling in regulating multiple aspects of intestinal development, homeostasis and cancerogenesis.

Material and Methods

-Probes

The following probes utilized in this study were described elsewhere. *Wnt-1* (86;180); *Wnt-2, Wnt-4, Wnt-5b, Wnt-6, Wnt-7a, Wnt-7b* (205); *Wnt-3, Wnt-3a* (240); *Wnt-8a* (241); *Wnt-8b* (241;242); *Wnt-9a/14, Wnt-9b/14b* (242;243); *Wnt-10a, Wnt-10b* (244); *Wnt-11* (245); *Fz-4 , Fz-7* (246); *Dkk-1* (247), *Lef* (194). Other probes were derived from RT-PCR products and correspond to the following nucleotides: 781-1640 for *Wnt-16*; for 1488-1815 for *Fz-1*, for 1245-1784 *Fz-2*; for 1371-1733 *Fz-3*; 1500-2500 for *Fz-5*; for 418-940 *Fz-8*; for 1296-1864 *Fz-9*; for 994-1413 *Fz-10*. The remaining probes correspond to ESTs obtained

from the IMAGE consortium. The Genbank accession numbers for these probes are the following: *Wnt-5a* (4317623), *Fz-6* (4973661), *sFRP-1* (BG975485), *sFRP-2* (BQ958218), *sFRP-3* (AA399912), *sFRP-4* (BC034853), *sFRP-5* (BC032921), *Dkk-2* (BE310466), *Dkk-3* (BQ933260), *Dkk-4* (BC018400), *WIF* (BG246236), *Cerberus* (AA120122), *TCF-4* (BG921346), *TCF-1* (BF141520) and *TCF-3* (AA015280). To insure the specificity of the probes we generated both sense and anti-sense probes for *Wnt-3, Wnt-9b, Wnt-4, Wnt5b, Wnt-6, Fz-4, Fz-6, sFRP-1, sFRP-5, WIF, Dkk-2, Dkk-3, TCF-4, TCF-1, TCF-3* and tested these in parallel in our *in situ* hybridization protocol. Representative examples are shown in Supplementary Figure 1 (see http://www.niob.knaw.nl/researchpages/clevers/files).

-*In situ* hybridization

Intestines from normal or APC^{min} mice (C57Bl6, 3 months of age) were flushed and fixed overnight in Formalin. Samples were then dehydrated and embedded in paraffin, sectioned at 8µM and processed for hybridization as described below. Sections were dewaxed, rehydrated, treated with 0.2N HCL, digested in proteinase K solution, post-fixed, treated in acetic anhydride solution and hybridized overnight for 24-48hrs at 68°C with various probes in 5X SSC (pH 4.5), 50% Formamide, 2% Blocking Powder (Roche), 5mM EDTA, 50µg/ml yeast tRNA, 0.1% Tween 20, 0.05%CHAPS and 50µg/ml Heparin. Sections were then rinsed in 2X SSC and washed for 3X 20minutes at either 60 or 65°C in 2X SSC/50% Formamide. Following several rinses in TBST, sections were then blocked for 1/2 hour in TBST 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 TBST, the color reaction was performed with NBT/BCIP solution. For image analysis, sections were temporally mounted in glycerol or permanently mounted after dehydration in Pertex. A complete protocol will be provided upon request. *In vitro* transcription reactions to generate labeled probe was performed as follows: 1µg of linearized DNA was incubated at 37°C for more than 2 hours with 4 µl transcription buffer (Promega), 2 µl of DTT 0.1M (Promega), 2 µl of Dig RNA labeling mix (Roche), 1 µl RNAse inhibitor (Promega) and 1.5 µl of T7 or T3 or SP6 (Promega) in a total volume of 20 µl.

-Antibody staining

Immunohistochemistry procedure is described elsewhere (86). Briefly, sections were pretreated with peroxidase blocking buffer (120 mM Na_2HPO_4, 43 mM citric acid, 30 mM NaN_3, 0.2% H_2O_2; pH 5.8) for 20 minutes at room temperature. Antigen retrieval was performed by boiling samples in Na-citrate buffer (10 mM, 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% BSA in PBS overnight at 4°C. The primary antibodies used in this study were rabbit anti-lysozyme (1:500; DAKO) and goat anti-TCF-4 (1:500; Santa Cruz). Rabbit anti-goat (DAKO) and rabbit EnVision+ (DAKO) were used as secondary antibodies.

CHAPTER 4

Wnt signaling induces maturation of Paneth cells in intestinal crypts.

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Wnt signaling induces maturation of Paneth cells in intestinal crypts.

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Abstract

Wnt signaling, transduced through β-catenin/Tcf4, maintains the undifferentiated state of intestinal crypt progenitor cells. Mutational activation of the pathway initiates the adenoma-carcinoma sequence. While all other differentiated epithelial cells migrate from the crypt onto the villus, Paneth cells home towards the source of Wnt signals, i.e. the crypt bottom. We find that expression of a Paneth gene program is critically dependent on Tcf4 in embryonic intestine. Moreover, conditional deletion of the Wnt receptor Frizzled-5 abrogates expression of these genes in Paneth cells in the adult intestine. Conversely, adenomas in APC mutant mice and colorectal cancers in man inappropriately express these Paneth cell genes. These observations imply that Wnt signals in the crypt can separately drive a stem-cell/progenitor gene program and a Paneth cell maturation program. In intestinal cancer, both gene programs are activated simultaneously.

Wnts induce Paneth cell maturation

Wnt signaling plays a key role in the intestinal epithelium (87). Central to this signaling pathway is the stabilization of β-catenin and its interaction with TCF transcription factors within the nucleus (39;40). Cytosolic levels of β-catenin are tightly regulated. In the absence of Wnt signals, a dedicated complex of proteins including APC, Axin and GSK3-β phosphorylates β-catenin, resulting in its ubiquitination and degradation by the proteasome (248;249). Signaling by Wnt factors inhibits the APC complex. As a result, β-catenin is stabilized and translocates into the nucleus where it interacts with nuclear Tcf transcription factors to drive the transcription of specific target genes (39;40). Mutational activation of the Wnt signaling pathway in intestinal epithelial cells inappropriately activates Tcf4 and initiates adenoma formation (10;11). We have recently determined the Tcf4 target gene program in colorectal cancer cells and have found that it is physiologically expressed in the proliferative crypt progenitors of the intestinal epithelium (69). As an integral part of this program, β-catenin and Tcf4 control expression of EphB cell sorting-receptors. These receptors allow the correct positioning of epithelial cells in a Wnt gradient along the crypt-villus axis (86).

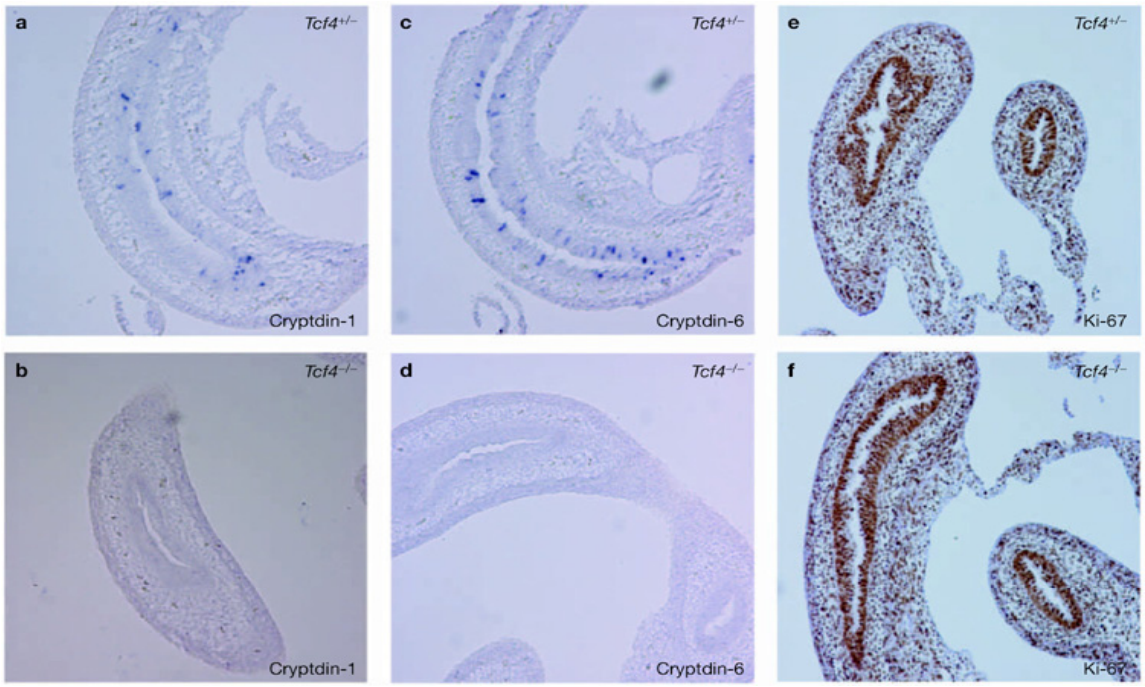

Figure 1. Cryptdins are target genes of the Tcf4-β-catenin signaling pathway.
Cryptdin-1 and Cryptdin-6 mRNA is readily detectable in the small intestine of $Tcf4^{+/-}$ E14.5 embryos (A, C), but is absent in $Tcf4^{-/-}$ embryos (B, D). No differences in proliferation were noted at E14.5 between $Tcf4^{+/-}$ and $Tcf4^{-/-}$ intestines as revealed by immunostaining for the proliferation marker Ki67 (E and F respectively).

Late embryonic mice deficient for the Tcf4 transcription factor fail to maintain the epithelial stem-cell compartments in the intervillus pockets of the small intestine (66). We utilized this genetic model to ask, by DNA array analysis, which genes are regulated by Tcf4 in the developing intestine. To this end, we compared expression profiles of $Tcf4^{-/-}$ and $Tcf4^{+/-}$ small intestines at E15.5, E16.5, and E18.5. As predicted, we identified multiple Tcf4 target genes (e.g. Myb, c-Myc, GPX-2, CDX-1) that were previously defined by inducibly blocking

the Wnt cascade in human colorectal cancer cell lines (69). Remarkably however, of the top-50 down-regulated genes, at least 8 encoded Paneth cell markers including several Cryptdins, Peptidoglycan Recognition Protein (PGRP) and Mpgc60 (see online version for Supplementary information, Table S1) (250-252). We realized that two previously published Tcf4 target genes in colorectal cancer cells, MMP7/Matrilysin (253) and EphB3 (86), also represent Paneth cell markers.

Paneth cells, which physically appear two weeks after birth, localize to the base of the crypts of Lieberkühn. They contain large apical secretory granules filled with a diverse array of antimicrobial proteins and peptides, including lysozyme and Cryptdins (254). The mouse Cryptdin gene family encodes at least 19 different Cryptdin proteins (255). In the small intestine, the Cryptdin-1 to -6 peptides are specific to Paneth cells (255). Cryptdin precursors are processed and activated in Paneth cells by the matrix metalloproteinase MMP-7 (256). Indeed, MMP-7-deficient mice do not process procryptdin precursors, resulting in a lack of mature Cryptdins and a defect in clearing intestinal infections (257). Below, we refer to the Tcf4-driven Paneth cell program as the MMP-7/Cryptdin program.

Despite the fact that Paneth cells are morphologically absent in embryonic and neonatal mice, the analyzed Cryptdin-1 and -6 mRNAs were readily detectable in the small intestine of $TCF4^{+/-}$ E14.5 embryos (Fig. 1A, 1C), yet absent from the small intestines of $Tcf4^{-/-}$ E14.5 embryos (Fig. 1B, 1D). Of note, the previously described $Tcf4^{-/-}$ phenotype, i.e. the abrogation of proliferation in the small intestinal epithelium, first becomes evident at E16.5 (66). Indeed, no differences were observed in expression of the cell cycle marker Ki67 at E14.5 (Fig. 1E, 1F).

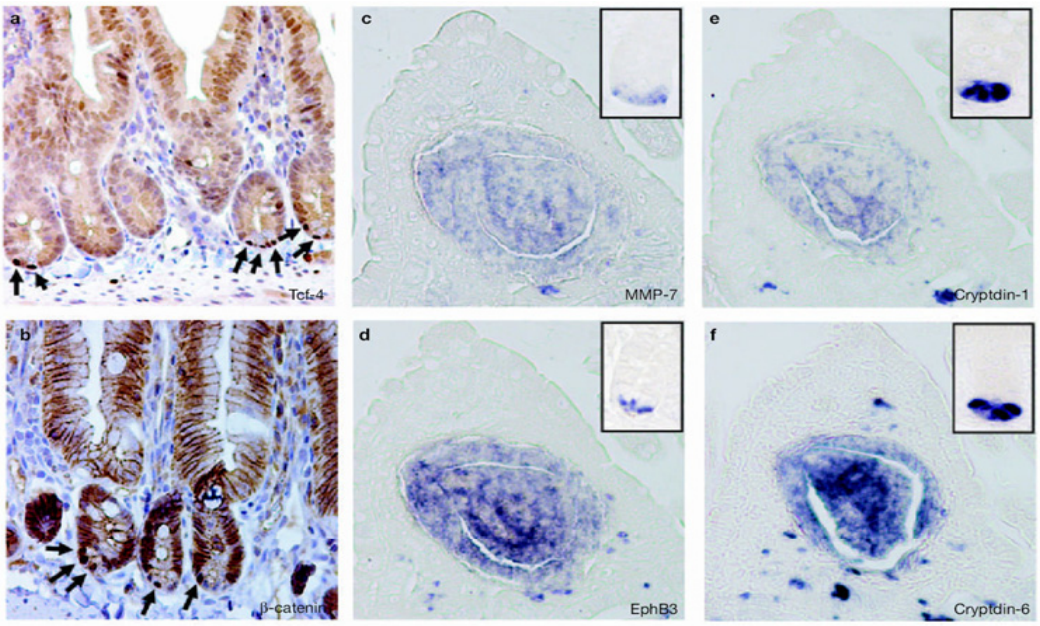

Figure 2. Strong nuclear expression of β-catenin and Tcf4 in Paneth cells and the upregulation of Paneth cell specific genes in APC^{min} tumors.
Immunohistochemical analysis revealed that the nuclei of Paneth cells contained high levels of the Wnt effectors Tcf4 (A) and β-catenin (B). *In situ* hybridization on the intestines of APC^{min} mice revealed that the Paneth cell genes MMP7 (C and (253)*)*, EphB3 (D and (86)) Cryptdin-1 (E) and Cryptdin-6 (F) are inappropriately expressed in adenomas. Inserts show normal Paneth cell expression of these Wnt targets in the intestinal crypt.

We have previously shown that proliferative crypt cells accumulate nuclear Tcf4 and β-catenin, indicative of active Wnt signaling. Similar analyses revealed that the nuclei of Paneth cells contained even higher levels of both Wnt effectors (Fig. 2A and 2B). Moreover, adenomas in APC mutant mice inappropriately expressed the MMP-7/Cryptdin program, including the genes encoding MMP7, EphB3, Cryptdin-1 and Cryptdin-6 (Fig 2C-2F). In addition, DNA array analysis on human colorectal cancers revealed that in the majority of these colon tumors (6 out of 8), the human functional counterparts of the Paneth cell-specific Cryptdins, Defensin-5 and Defensin-6 were also highly up-regulated (28.0 and 44.5 times, respectively).

The combined data suggested that the MMP-7/Cryptdin program contained direct Wnt target genes. We have previously determined a 12 bp optimal TCF binding site conserved from fly to man, AAGATCAAAGGG, where changes are tolerated in the first three bases only (258;259). Alignment of the promoters of murine Cryptdin-1, -2, -3, -5 and -6 revealed the complete conservation of a high-affinity TCF site, AAcATCAAAGGG (see Supplementary information Fig. S1). The promoters of human Defensin-5 and -6 also contain, at a conserved position, a high-affinity TCF-binding site fitting this consensus, AgcATCAAAGGG (see Supplementary information Fig. S1). A recent transgenic study has demonstrated that a 1402 bp promoter fragment of the human Defensin-5 gene faithfully drives Paneth-cell specific gene expression (260). A transient promoter assay demonstrated that this Defensin-5 promoter is indeed activated by β-catenin associated with endogenous Tcf through the conserved TCF site (Fig. 3A). The activation could be blocked by dominant-negative Tcf4 (Fig. 3A), while mutation of the conserved Tcf binding site in the Defensin-5 promoter also abrogated the activation by endogenous Tcf associated with β-catenin (Fig. 3B). Similarly, the murine Cryptdin-1 and Cryptdin-6 promoter were activated by the β-catenin/Tcf bipartite transactivation complex (Fig. 3C).

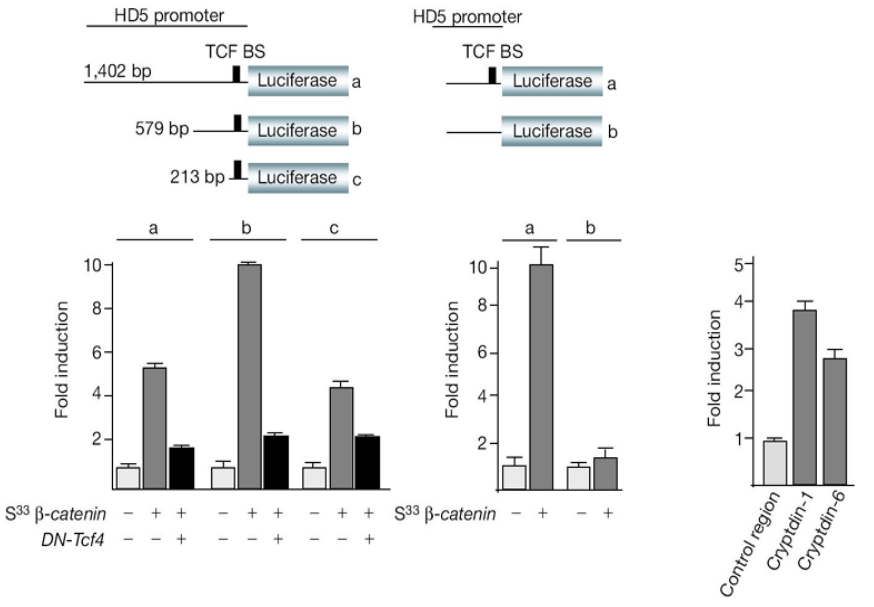

Figure 3. Tcf/β-catenin activation of the Cryptdin-1, Cryptdin-6 and Defensin-5 promoter through a highly conserved TCF binding site. Reporter construct containing deletions of the Paneth cell specific Defensin-5 promoter (260), containing a conserved TCF binding site (AAGATCAAAGGG) were generated. In a transient transfection assay, the Defensin-5 promoter constructs could be activated by an active Tcf/β-catenin bipartite transcription complex, which could be blocked by a dominant negative Tcf4 (A). The mutation of the highly conserved Tcf binding site (AAGATCCCCGGG) in the Defensin-5 promoter abrogated this Tcf/β-catenin

signaling (B). Reporter constructs of Defensin-5, Cryptdin-1 and Cryptdin-6 containing the highly conserved TCF binding site, could be activated by an active Tcf/β-catenin bipartite transcription complex (C). Chromatin immunoprecipitation analysis of the Cryptdin-1 and Cryptdin-6 promoters in Paneth-cell enriched crypt fractions revealed the occupancy of β-catenin. Bars represent Real-Time PCR values obtained with primers spanning the respective promoters normalized to the inputs and values obtained with primers spanning a control region downstream of the Cryptdin-6 gene, which was set to 1 for each calculation.

We also performed chromatin immunoprecipitations with an antibody directed against β-catenin on isolated mouse crypts (261). The results unambiguously demonstrated the presence of β-catenin on the promoters of Cryptdin-1 and -6 (Fig. 3), confirming the notion that these genes represent direct Wnt targets.

Independent of these experiments, we analyzed mRNA expression of all Frizzled family members in fetal and adult intestine by Northern blotting and *in situ* hybridization (A.G., submitted). In adult small intestine, we detected crypt-specific expression of 3 *Wnt* genes (*Wnt3*, and *Wnt9b*). Moreover, *Frizzled5 (Fz5)* and *Frizzled6* were prominently expressed in small intestinal epithelium. Interestingly, *Frizzled6* was expressed by all epithelial cells with the exception of Paneth cells, while Fz5 was expressed by all cells in the crypt. $Fz5^{-/-}$ embryos die *in utero* around E10 due to defects in yolk sac and placental angiogenesis (262). To analyze the function of *Fz5* in the adult intestine, we created a mutant *Fz5* allele, in which the single exon-open reading frame was sandwiched by LoxP sequences (see Supplementary information Fig. S2). We then generated germline chimeras and crossed their offspring with the intestine-specific K19-Cre knock-in mouse (263). The K19-Cre allele is expressed in a somewhat mosaic pattern throughout the intestinal epithelium from early stages of embryonic development into adult life (263). This was confirmed by the generation and subsequent analysis of a K19-LacZ knock-in mouse. This analysis revealed patchy expression of LacZ along the gastrointestinal tract (see Supplementary information Fig. S3). The expression of LacZ was highest in the duodenum and gradually declined along the gastrointestinal tract towards the rectum.

$Fz5^{LoxP/LoxP}$*K19-Cre* mice were healthy and fertile. Histological analyses of the entire gastrointestinal tract revealed a single abnormality. While the proliferative crypt compartment was intact, Paneth cells were randomly distributed in crypts and villi, as evidenced by morphology and by the markers lysozyme (Fig. 4D), FAS ligand (Fig 4E) and staining for Zinc-positive granules by the pholoxine tartrazine technique of Lendrum (Fig. 4E) (254;264;265). The highest number of miss-positioned Paneth cells occurred in the duodenum (not shown), coinciding with the expression of the Cre enzyme. The phenotype of miss-positioned Paneth cells was indistinguishable from that of $EphB3^{-/-}$ mice (86). Indeed, the miss-positioned Paneth cells of the $Fz5^{LoxP/LoxP}$*K19-Cre* mice never expressed the Tcf4 target gene *EphB3*, while the crypt Paneth cells did express EphB3 (Fig. 4G), providing an explanation for the observed miss-positioning and confirming our notion that the Wnt cascade provides positional clues along the crypt/villus axis (86). The miss-positioned Paneth cells appeared immature, based on the small size of their granules relative to those of Paneth cells in the crypts. These observations suggested that Fz5 plays a non-redundant role in the

transduction of canonical Wnt signals in the Paneth cell maturational process. Indeed, the miss-positioned Paneth cells displayed a complete absence of nuclear β-catenin (Fig. 4H). *In situ* hybridization revealed the absence of Cryptdin-1 mRNA in miss-positioned Paneth cells (Fig. 4L). Of note, the expression of the Tcf4-independent Paneth cell markers lysozyme remained unchanged, allowing for unambiguous identification of the Paneth cells (Fig. 4C, 4D, 4I, 4G and 4K resp.). In crypts of $Fz5^{LoxP/LoxP}$-*K19Cre* mice, some Paneth cells remained at the crypt base and expressed targets of the Wnt pathway. We reasoned that this resulted from the inefficiency of Cre-mediated deletion, due to the mosaic expression pattern of the Cre enzyme in these mice (263). Indeed, staining of the intestine derived from the $Fz5^{LoxP/LoxP}$-*K19Cre* mice revealed the presence of Fz5 expressing Paneth cells in the crypt, while all miss-positioned Paneth cells were Fz5-negative (Fig. 4F). In conclusion, the Wnt signaling pathway activates the MMP-7/Cryptdin maturation program in Paneth cells through the Fz5 receptor.

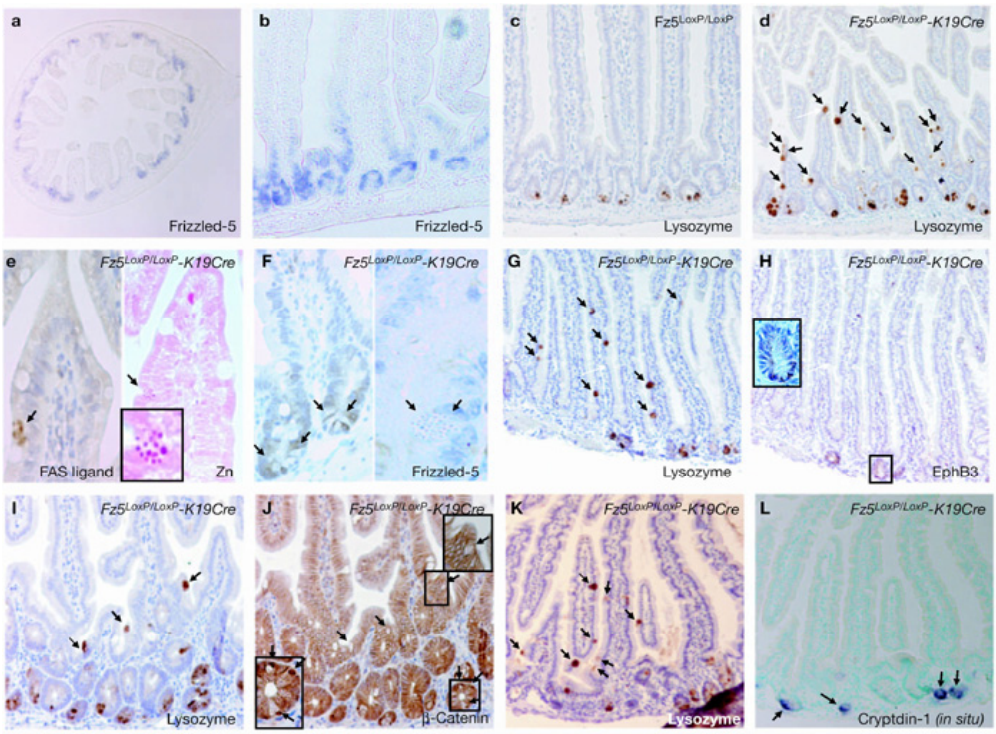

Figure 4. Paneth cells in the small intestine of $Fz5^{LoxP/LoxP}$ K19-Cre mice.
In situ hybridization with a Fz5 probe revealed that Fz5 is expressed in the (developing) crypts of neonatal (A) and adult mice (B). Staining of the intestine derived from $Fz5^{LoxP/LoxP}$ mice (C) and $Fz5^{LoxP/LoxP}$ K19-Cre mice (D-L). The panels G and H; I and J; and K and L represent three sets of serial sections. The panels C, D, G, I and K were stained with an antibody directed against lysozyme. Panel E were stained with an antibody directed against the FASligand and stained for Zn, Panel F with an antibody directed against Fz5, Panel H with an antibody directed against EphB3, Panel J with an antibody directed against β-catenin, and panel L with an *in situ* probe directed against Cryptdin-1. The Paneth cells of the $Fz5^{LoxP/LoxP}$ K19-Cre mice, in sharp contrast to the Paneth cells of $Fz5^{LoxP/LoxP}$ mice (C), stray from the crypt (B). Panel E shows that the mislocoalized Lysozyme positive cells also stained for the Fas ligand and contains Zinc-positivity of cytoplasmic granules. These markers unequivocally identify the mislocalized cells as Paneth cells. Panel shows that the mislocalized Paneth cells do not express Fz5, while the Paneth cells which stay at the bottom still express Fz5. The miss-positioned Paneth cells express the Paneth cell marker lysozyme (G, I, K), but lack expression of the Wnt signal transducer nuclear

β-catenin (J) or the Wnt target genes EphB3 (H), Cryptdin-1 (L) or Cryptdin-6 (results not shown). Insets in J show that the Paneth cells in the crypt do contain β-catenin in the nucleus (bottom left), whereas miss-positioned Paneth cells do not have nuclear β-catenin (top right).

The canonical Wnt signaling cascade is known to control multiple biological phenomena in vertebrates and model organisms, involving either cell fate determination or maintenance of stem/progenitor cells in compartments with predefined fates (64;174;266;267). A recent example of the latter is the role of Wnt signaling in the hematopoietic stem cell (268). The effect of Wnt signaling on Paneth cell maturation described here contrasts with this paradigm. In the absence of Wnt signals, Paneth cells are correctly specified, but fail to express the MMP-7/Cryptdin program. As a consequence, these postmitotic cells do not undergo morphological maturation. This adds a twist to the central role that the Wnt cascade plays in the biology of the crypt. Wnt signals near the bottom of crypts are crucial for the maintenance of the undifferentiated progenitors. Postmitotic immature Paneth cells, once specified, appear to utilize Wnt signals for the opposite aim: to reach full maturity through the Wnt-induced expression of the MMP-7/Cryptdin program.

Methods.

DNA array analysis.

DNA array analysis of mouse embryos. Total RNA was isolated from small intestine of $Tcf4^{+/-}$ and $Tcf4^{-/-}$ embryos (E15.5, E16.5 and E18.5) using TRIzol Reagent (Gibco BRL Life Technologies). cDNA synthesis and labeling were performed according to Affymetrix guidelines. Samples were hybridized on Murine Genome U74 arrays (Affymetrix) representing 36.000 full length genes and ESTs. Overall fluorescence for each GeneChip was scaled to a target intensity of 200 and pairwise comparisons were performed with Affymetrix MICROARRAY SUITE. Genes were selected based on the following criteria. For each time point, GeneChips for Tcf4 wt intestines were designated as baseline and all mRNAs which were decreased in $Tcf4^{-/-}$ intestines more than 1.5 fold in two out of three time points were selected.

DNA array analysis of human tumors. Detailed protocols and results will be published elsewhere (Hlubek et al., manuscript in preparation). In brief total RNA was separately prepared from eight microdissected human colorectal carcinomas and normal mucosa. Probe labelling and hybridisation to Affymetrix U133A arrays, representing 14,500 genes, were performed according to the Affymetrix guidelines (Affymetrix, Santa Clara, CA). The arrays include a set of human maintenance genes (> 100 probe sets) to facilitate the normalization and scaling of detected fluorescence values. This set of genes served as a tool to normalize expression levels, and allowed quantitative comparison of different tissues. Mean expression values of 8 tumours and normal colon mucosa were used to calculate the tumour/normal ratio of the selected genes.

Promoter analysis.

Schematic of the Defensin-5 deletion promoter constructs cloned in Pgl3basic (Promega). HEK293T cells were cotransfected with 500 ng of the indicated HD-5 promoter

constructs and/or plus 10 ng S^{33}β-catenin expression vector and/or 500 ng of a DN-Tcf4 expression vector and 50 ng of TK-Renilla reporter vector using Fugene (Roche). Total amounts of plasmid were kept constant by adding the empty DNA vector. The assays were harvested 20 hours later to assess luciferase and renilla activity using the dual-luciferase reporter assay (Promega). The assays were performed in triplicate and repeated at least three times. Data reported are normalized for transfection efficiency.

Chromatin Immunoprecipitation analysis on isolated crypts.

The Paneth-cell enriched crypt fractions were cross-linked with 1% formaldehyde for 10 min at room temperature, with gentle agitation. Cross-linking was terminated by the addition of glycine to a final concentration of 0.125M. The cells were washed twice with ice-cold PBS and swelled on ice for 10 min in 25 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5% NP40, 1 mM DTT and protease inhibitor cocktail (Roche). After Dounce homogenization the nuclei were resuspended in sonication buffer containing 50 mM HEPES (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.5% SDS and protease inhibitors, sonicated for 4 min with 30 sec on and 2 min off cycles at high settings in a Diagenode Bioruptor to produce fragments with an average length of 400 bp. The chromatin was centrifuged, adjusted to 0.1% SDS, precleared and subjected to immunoprecipitation as described (261), with an antibody directed against β-catenin (BD Transduction Laboratories). The DNA in the immunoprecipitates was analyzed in SYBRGreen Real-Time PCR reactions on a MyiQ apparatus (Bio-Rad). The primers used for the PCR amplifications were the following: Cryptdin-1 promoter, sense: 5'-GGGAAATGGGAGTAGACTGAAG-3' and antisense: 5'-GGAAGTTGGAGAGGCTGTTAC-3', Cryptdin-6 promoter, sense: 5'-GATGGGAA ATGGGAGAAGACTG-3' and antisense: 5'-GGGAAGTTGGAAAGGGTGTTAC-3', control region downstream Cryptdin-6, sense: 5'-GCCATTCGGATGTTCACTCTG-3' and antisense: 5'- TCGTCTTGACCAACTGTTCT TG-3'.

Generation of the Cre inducible Fz5 KO mouse.

A P1 clone containing the mouse Fz5 gene was isolated from a mouse genomic library. The Fz5 coding region was PCR amplified (5'cgg gat cca gta ctg aat tcg ggc gtc aca ctc aag act cc3' and 5'cgc gga tcc aac agt aac ctc att aca atg cc3') and cloned between 2 LoxP sites of the modified pFlox vector. The 1 Kb 3'arm was subsequently cloned, followed by cloning of the 2.5 Kb 5' arm. Details of this construct are available on request. The construct was linearized and electroporated into E14 ES cells. Approximately 300 ES cell clones selected by neomycine (250 μg ml) were screened by Southern-blot analysis for the presence of a recombinant 2.4-kb *Eco*RI band, in addition to the endogenous 4.5-Kb fragment. A 350 bp PCR amplified fragment was used a probe (5'gat act agc acg tct gtc acc3' and 5'ccc cgc ggc ccc gcc cgg ag3'). Four out of 300 ES cell clones analyzed had undergone correct integrations at both ends of the construct. Chimaeric mice were derived from two randomly chosen clones; both transmitted the mutation through the germline.

Tissue sample preparation, immunohistochemistry and in situ hybridisation.

The intestinal tract was dissected as a whole and flushed gently with cold PBS to remove any faecal content. The small intestine was rolled up into a compact circle and fixed in Formalin at RT for 16 hours. The tissues were sectioned (2–6 μm). Following dewaxing and hydration, sections were pretreated with peroxidase blocking buffer (120 mM Na_2HPO_4, 43 mM citric acid, 30 mM NaN_3, 0.2% H_2O_2; pH 5.8) for 15 minutes at room temperature. Antigen retrieval was performed by boiling samples in Na-citrate buffer (10 mM, pH 6.0). After 20 minutes, the boiling pan was allowed to slowly cool down to room temperature. Incubation of antibodies was performed in BSA in PBS overnight at 4°C for antibodies directed against Cryptdin, Tcf4, Fz5 and MMP-7 and at RT for 1 hour for antibodies directed against lysozyme and Ki67. In all cases, the Envision$^+$ kit (DAKO) was used as a secondary reagent. The incubation time was 30 min. For goat antibodies, a bridge step using rabbit anti-goat antibodies was required. Stainings were developed using DAB. Slides were then counterstained with hematoxylin and mounted. The Fixation and staining of sections used for β-catenin and EphB3 expression was exactly as described (86). The following antibodies were used. Goat anti-EphB3 (1:100; R&D systems), Rabbit anti-lysozyme (1:1500; DAKO), Mouse anti-Ki67 (1:100; Novocastra), and mouse anti-β-catenin (1:50; Transduction Labs), Mouse anti-Tcf4 (1:400; Signal Transduction Lab), Goat anti-MMP-7 (1:50; R&D systems), Rabbit anti-Cryptdin-1 (1:1500; kind gift of Dr. Ouellette (269)), Rabbit anti-Frizzled5 (1:100; Upstate). *In situ* hybridizations were performed as described (270). As a probe for Cryptdin-1 and -6 we used anti-sense RNA derived from the Image clones 1096215 and 1545534 resp.

Wnts induce Paneth cell maturation

CHAPTER 5

Tcf4 promotes secretory lineage commitment via Math1 and represses enterocyte differentiation during intestinal development

To be submitted

Tcf4 promotes secretory lineage commitment via Math1 and represses enterocyte differentiation during intestinal development

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Abstract

In the intestinal epithelium, the Wnt cascade promotes cycling of progenitor cells and concomitantly drives maturation of secretory cell lineages. Despite this knowledge, the precise mechanism by which Wnt signaling coordinates both proliferation and differentiation of gut epithelial cells has remained largely unexplored. To gain insight into the dual role of Wnt signaling, we examined the gene expression profiles of wild-type vs *Tcf4-/-* fetal guts. In Tcf4 deficient intestines, we found specific upregulation of enterocytic markers (ie. *Fabp1, Creb3l3, Nr1h4,* etc.) along with downregulation of secretory lineage markers (ie. *Tff3, Chromogranin B, and Spink4,* etc.) and crypt progenitor markers (ie. *c-Myc, c-Myb, TcfAP4,* etc). Further analysis suggested that Tcf4 promotes early commitment of secretory lineages through activation of the basic helix-loop-helix transcription factor, Math1. Moreover we found that Tcf4-mediated effects on cell fate were independent of any changes in the expression of Hes family members. Finally our results imply a model whereby Tcf4 coordinates renewal of progenitor cells, repression of enterocyte differentiation and commitment towards secretory lineages via Math1.

Introduction

The intestinal epithelium is an ideal model system in which to dissect the mechanisms regulating epithelial cell proliferation and differentiation (271). The proliferative compartment of the gut epithelium is composed of a population of multipotent progenitor cells. In the adult intestine, progenitor cells reside in the crypts of Lieberkühn, while in the fetal intestine these cells occupy so-called intervillus pockets. As progenitor cells mature they give rise to two differentiated lineages, absorptive and secretory cells. The absorptive lineage or enterocytes functions in nutrient uptake and comprise the vast majority of epithelial cells lining the intestine. The secretory lineage can be divided into cells producing: mucus (i.e.goblet cells), hormones (ie. enteroendocrine cells) and anti-microbial peptides (ie. Paneth cells). Most differentiated cell types cover the finger-like projections termed villi. One notable exception to this rule includes the Paneth cells, which complete their differentiation program at the very bottom of the crypts.

The self-renewing capacity of the intestinal epithelium strongly depends on continual activation of the Wnt cascade. Secreted Wnt factors induce their biological effects in part by triggering the release of β-catenin from a destruction complex comprised of APC, GSK3β and Axin, which in the absence of Wnts, promotes ubiquitin-mediated degradation of β-catenin (for up-to-date reviews see www.stanford.edu/~rnusse/ wntwindow.html). Subsequently, β-catenin translocates to the nucleus where it associates with members of the Tcf family of transcription factors (Tcf1, Tcf4, Tcf3 and Lef) and thereby activates transcription of target genes (39;40). Several *in vivo* models have highlighted the importance of Wnt signaling components in driving proliferation of the progenitor cells in the intestinal epithelium. Aberrant activation of the Wnt pathway through mutations in the genes encoding for APC, β-catenin or Axin2 leads to hyperproliferation and ultimately colon cancer (9;11;73;128;129). Moreover, perturbed Wnt signaling resulting from inactivation of *Tcf4*, *β-catenin* or ectopic expression of the Wnt antagonist, Dkk-1 results in the loss of proliferative cells (65-68).

Analysis of the above mouse models has also led to the notion that Wnts specifically promote the formation of secretory lineages (65;66;68). The earliest known determinant of secretory cells is the basic helix-loop-helix transcription factor Math1. Targeted deletion of *Math1* results in a total loss of secretory cell types (95). Another key cell fate determinant in the intestine is the Notch target gene *Hes1*. Functional evidence from neuronal and intestinal cells indicates that Hes1 appears to block *Math1* transcriptional activation and thereby skews progenitor cells away from secretory lineages. Indeed, inactivation of *Hes1* results in a relative increase in the numbers of secretory cells when compared to enterocytes (96;98). Similarly, blockage of the Notch pathway (and presumably Hes1) results in a massive conversion of progenitor cells into postmitotic goblet cells (272;273); and conversly constitutive activation of the Notch receptor results in a perturbed production of secretory cells (274-276). It is currently unknown whether Wnt signaling promotes secretory lineage differentiation by modulating Math1/Hes1 activity or whether Wnts act via an independent mechanism.

To assess how maintenance of progenitor cells and differentiation towards secretory cells types is controlled by Wnt signaling, we wished to identify and characterize Wnt responsive genes in the intestine. By DNA microarray analysis, we compared the gene expression profiles of wild-type and *Tcf4* deficient fetal small intestines. Previously using this approach, we reported that Tcf4 regulates the expression of numerous Paneth cell markers including the anti-microbial peptides, cryptdins (103). Herein, we present the complete list of Tcf4 responsive genes including novel crypt progenitor markers, as well as secretory cell and enterocytic markers. Finally, we propose a model whereby Tcf4 drives secretory lineage commitment through Math1 upregulation and actively represses enterocyte differentiation via a Hes1 independent mechanism.

Materials and Methods

Immunohistochemistry

Fetal intestines were fixed overnight in 4% PFA at 4°C and adult intestines were fixed overnight in Formalin at room temperature. Samples were then dehydrated, embedded in paraffin and sectioned at 4μM. Antigen retrieval and antibody stainings were carried out as detailed in (277). The primary antibodies used in this study were mouse anti-Ki67 (1:100; Novocastra), rabbit anti-Math1 (1:50 dilution) and rabbit anti-synaptophysin (1:200 dilution; DAKO). The EnVision+ system (DAKO) was used as a secondary antibody.

In situ hybridization

Intestines were fixed and embedded in paraffin and sectioned at 8μM. Sections were dewaxed, rehydrated, treated with 0.2N HCl, digested in proteinase K solution, post-fixed, treated in acetic anhydride solution and hybridized overnight for 24-48hrs at 68°C with various digoxigenin -labeled RNA probes in 5X SSC (pH 4.5), 50% Formamide, 2% Blocking Powder (Roche), 5mM EDTA, 50μg/ml yeast tRNA, 0.1% Tween 20, 0.05% CHAPS and 50μg/ml heparin. Sections were then rinsed in 2X SSC and washed for 3X 20 minutes at either 60 or 65°C in 2X SSC/50% formamide. Following several rinses in TBST, sections were then blocked for 1/2 hour in TBST containing 0.5% Blocking Powder (Roche). Next, sections were incubated in blocking solution overnight at 4°C with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1/2000 dilution) (Roche). After washing several times in TBST, the color reaction was performed with NBT/BCIP solution. For fluorescence *in situ* hybridization (FISH) sections were pretreated as above except for the following modifications. Sections were hybridized with a digoxigenin-labeled *Tff3* probe and a FITC-labeled *Spdef* probe. Following post-hybridization washes sections were treated with peroxidase blocking buffer (1.5% H_2O_2 in PBS) for 30 minutes. Next, sections were blocked with 1% BSA in PBS for 30 minutes, followed by HRP-conjugated anti-FITC Fab fragments at room temperature for 2 hours (1:250) (Roche). Sections were washed 5X in PBS followed by treatments with Alexa Fluoro 488-labeled tyramide according to manufacturer's

instructions (Molecular Probes). Following a second peroxidase blocking step, sections were incubated with HRP-conjugated anti-digoxigenin Fab fragements at room temperature for 2 hours (1:250) (Roche). After washes sections were treated with Alexa Fluoro 546-labeled tyramide. Stainings were visualized using sequential scanning with a Leica TCS SP2 AOBS confocal microscope.

-Probes

The following probes utilized in this study correspond to ESTs obtained from the IMAGE consortium. The Genbank accession numbers for these probes are the following: *Vav3* (BQ887952), *Tcfap4* (BC047270), *Nr1h4* (BG971664), *FABP1* (BF384218), *Creb3l3* (BX521612), *Tff3* (BC011042), *Hop* (BQ936961), *Gob4* (BQ946802), *Gob5* (BQ946441), *Cholecystokinin* (AI385905), *Chromogranin B* (BU503622), *Spink4* (AA823442), *c-Myc* (BI080251), *Spdef* (BG916428), *Klf4* (BI692353). The Hes1 probe was described elsewhere (278).

-Microarrays

Total RNA was isolated from whole small intestines of E15.5 wt, *Tcf4+/-* and *Tcf4-/-* embryos using TRIzol Reagent (Invitrogen, Carlsbad, CA). Pooled RNA derived from two wild-type or two *Tcf4-/-* littermates were compared in duplicate experiments. Sample pairs were also dye-swapped and processed at the same time. cDNA synthesis and Cy-3 and Cy-5 labelings were performed using the Agilent low RNA input fluorescent linear amplification kits. Samples were hybridized on Whole Mouse Genome Oligo Microarrays (G4122A). Genes were selected based on the following criterion. All mRNAs that were decreased or increased by more than 1.5-fold in *Tcf4-/-* intestines in duplicate experiments were selected. The complete list of changed genes is available at: http://www.niob.knaw.nl/ researchpages/clevers/files/Completelist.xls

-RT-PCRs

As for microarrays, total RNA was isolated from the small intestine of E15.5 wt, *Tcf4*$^{+/-}$ and *Tcf4-/-* embryos using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 0.5µg of total RNA using random hexomers. PCRs were performed with the following oligos: *Hes1* (F: AACCTGCAGCGGGCGCAGATGAC, R: AATGGCGCGTGCTGGGGACCGG), *Hes5* (F: GGCTCCCTCCTCCGGCTGGCT, R: CGGGCTGGGGTGAGCCAACCC), *Hes6* (F: AGCCCCTGGTGGAGAAGAAGCGAC, R: TCGATGGCTTGGCACGTGGACACG), *Hes7* (F: CCTCCGGAACCCGAAGCTGGA, R: CGGTAGCCGTGCAGCGCGGAG), *Math1* (F: TCGCACCGCCTCCTCCTATGAAG, R: AGAGTCACTGTAATGAGAGTGGGGGG), *Ngn3* (F: GGAGCAGAGAGGCTCAGCTATCC, R: GTGCCAACTCGCTCTTGGGCCTG), *Pax4* (F: GCGCAGGCAAGAGAAGCTGAAATG, R: GATGGCACTTGTCCTGGGCCTC), *NeuroD* (F: GGACACGAGGAATTCGCCCACGC, R: TGCCTCGTGTTCCTCGTCCTGA), *beta-actin* (F: AAGAGAGGCATCCTCACCCT, R: TACATGGCTGGGGTGTTGAA)

Results

Loss of proliferative epithelial cells and secretory lineages in *Tcf4-/-* intestines

Tcf4-/- mice die postnatally presumably due to their inability to maintain proliferative cells in the small intestine. As shown previously (66) and in Figure1, immunohistochemical analysis of the proliferation marker Ki67 revealed a severe impairment of epithelial cell proliferation in *Tcf4-/-* small intestines starting at E16.5. However, at earlier time-points (E14.5 and E15.5), proliferation within the epithelial appeared unaffected. Tcf4 mutants also displayed a drastic reduction in the numbers of enteroendocrine cells and goblet cells in the small intestine (Figure 1I-L). Although not morphologically evident until after birth, Paneth cells also appeared to be absent in *Tcf4-/-* intestines based on the analysis of *Cryptdin-1* expression (data not shown). Note however that upon closer examination goblet cells were apparent, albeit at reduced numbers, towards the most distal regions of the small intestine (data not shown). Similarly enteroendocrine cells were also detected near the gastro-duodenal junction.

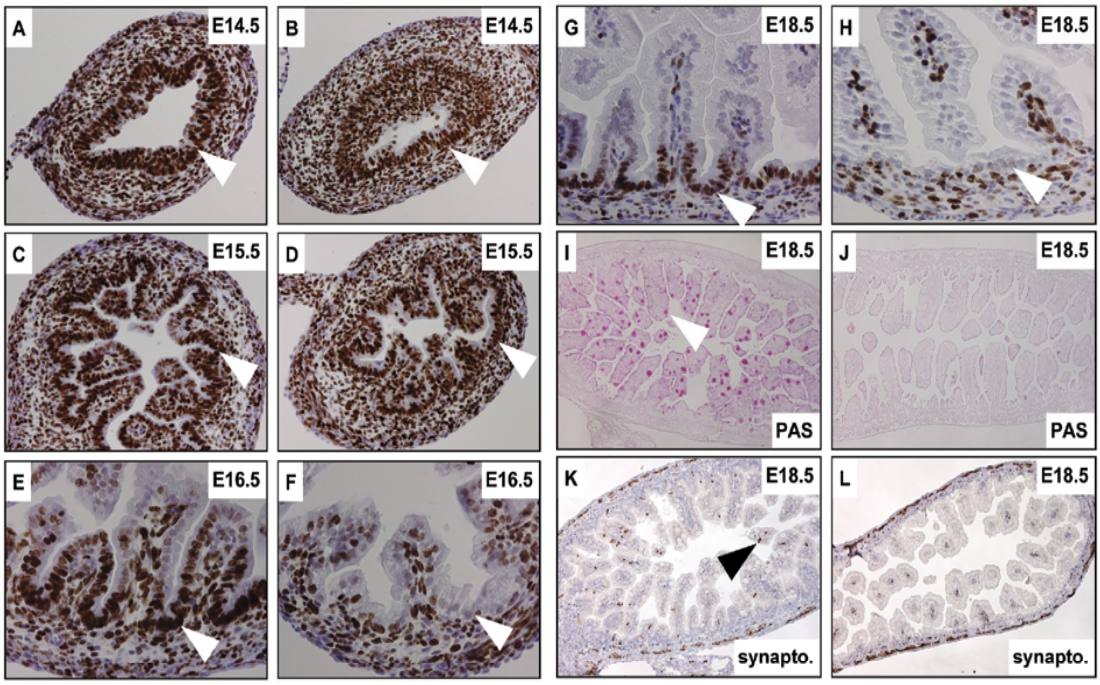


Figure 1. Loss of proliferative epithelial progenitors and secretory lineages in *Tcf4-/-*small intestines. Immunohistochemical analysis of proliferative cells, goblet cells and enteroendocrine cells at various developmental stages (E14-E18) in wild-type (A, C, E, G, I, K) and *Tcf4-/-* (B, D, F, H, J, L) small intestines. (A-H) Ki67 stainings show that prior to E16.5 proliferation throughout the epithelial lining (white arrowheads) is maintained in *Tcf4-/-*intestines. Periodic acid-Schiff (PAS) stainings (I and J) and anti-synaptophysin stainings (K and L) in E18.5 small intestines reveal a massive loss of goblet cells and enteroendocrine cells respectively in *Tcf4* mutant intestines.

In order to shed light on the genetic program regulated by Tcf4 during intestinal development, we performed microarray profiling on small intestines derived from normal or

Tcf4-/- littermates. We reasoned that the ideal developmental stage in which to carry out such analysis would be E15.5. At this stage proliferation within the epithelium is maintained in *Tcf4* knockouts; therefore changes in gene expression observed in *Tcf4* mutants are more likely to reflect the loss of *Tcf4* rather than the absence of cellular compartments, as would be the case when comparing intestines at later stages (E16.5 and beyond). E15.5 whole small intestines from two wild-type and *Tcf4-/-* intestines derived from two independent litters were harvested and profiled using Agilent Whole Genome Mouse arrays. As shown in supplementary Table I we selected genes, which were significantly ($p \leq 0.05$) up or downregulated at least 1.5 fold in *Tcf4-/-* intestines. Based on this criterion, we found over 100 genes downregulated and over 200 genes upregulated in *Tcf4-/-* guts. These genes were further categorized into functional groups based on their predicted or known expression patterns in the intestine (see complete list of genes at http://www.niob.knaw.nl/researchpages/clevers/files/Completelist.xls).

Enterocytic markers are upregulated in *Tcf4-/-* small intestines

As previously shown in our laboratory (69), inhibition of Wnt signaling in colon cancer cell lines results in upregulation of intestinal differentiation markers. To examine whether this observation holds true in the fetal small intestine we examined the list of upregulated genes in *Tcf4* mutant guts. As shown in Table I removal of Tcf4 resulted in an

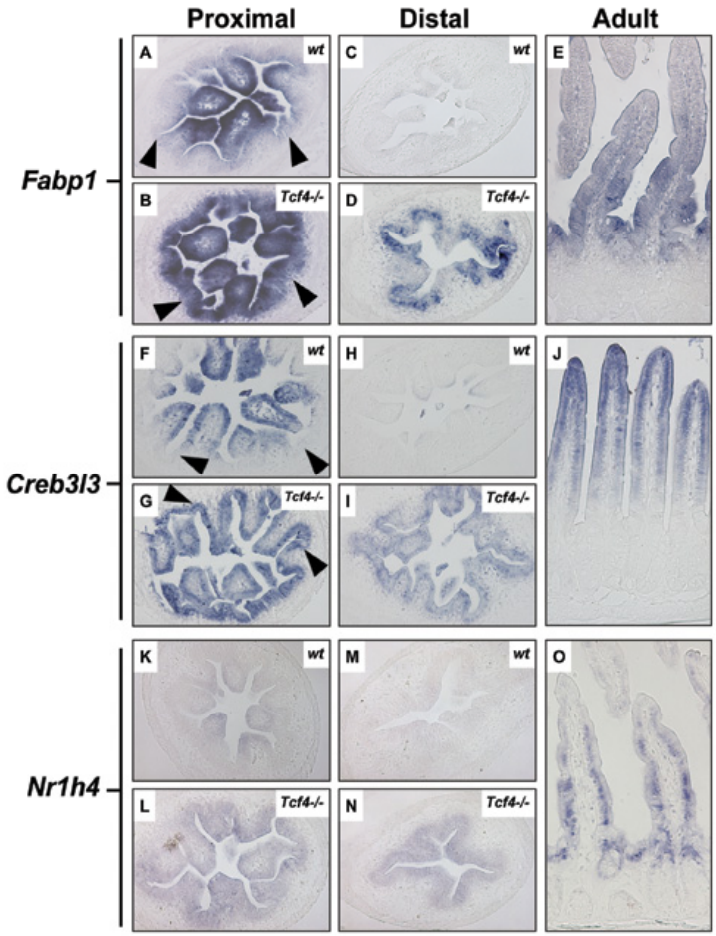


Figure 2. Upregulation of enterocyte markers in *Tcf4-/-* small intestines. *In situ* hybridization analysis of *Fabp1* (A-E), *Creb3l3* (F-J), *Nr1h4* (K-O) in wild-type and *Tcf4-/-* fetal intestines. Anterior and posterior regions of the fetal intestinal tract are depicted in panels (A, B, F, G, K, L) and panels (C, D, H, I, M, N) respectively. Expression levels of enterocytic markers are strongly upregulated in the distal *Tcf4-/-*intestine and also the intervillus regions (black arrowheads) of the proximal *Tcf4-/-* intestine. Panels (E, J, O) confirm that *Fabp1, Creb3l3, Nr1h4* are enterocytic markers in the mature intestine.

increase in genes specifically expressed in differentiated enterocytes (ie. *Apo4, Fabp1, Fabp2, Creb3l3,* etc.). To validate these findings we performed *in situ* hybridizations on sections of E15.5 wild-type and *Tcf4* mutant small intestines using probes specifically recognizing three enterocyte markers shown in Table I ie. *Fabp1, Creb3l3* and *Nr1h4*. As shown in Figure 2, in wild-type embryos all three markers were limited to the emerging villi in the anterior regions and were barely detectable in the distal regions of the intestine. However, in *Tcf4* mutant embryos *Fabp1, Creb3l3* and *Nr1h4* were strongly expressed in both propective villus and intervillus regions (Figure 2, see arrowheads), and where ectopically expressed in the distal regions of the intestine (Figure 2). Finally, in Figure 2E, J, O we showed that in the mature adult intestine *Fabp1, Creb3l3* and *Nr1h4* were confined to villus enterocytes and undetectable in crypts, indicating that these genes are indeed markers of fully differentiated enterocytes.

Loss of crypt progenitor markers in *Tcf4-/-* small intestines

Gene expression profiling in normal and colon cancer cells has shown that Wnt signaling promotes the expression of genes typically found in crypt progenitor cells (69;73). We now show based on our analysis of downregulated genes in *Tcf4-/-* intestines that, long before the formation of proper crypts, Tcf4 drives the expression of several known crypt progenitor markers (ie. *Sox9* (279) *, cMyc* (69)*, Gpx2*(280) *,c-Myb* (281) and *Axin2* (149), etc.). In Figure 3, we confirmed these findings by showing that both *c-Myc* and *c-Myb* expression was

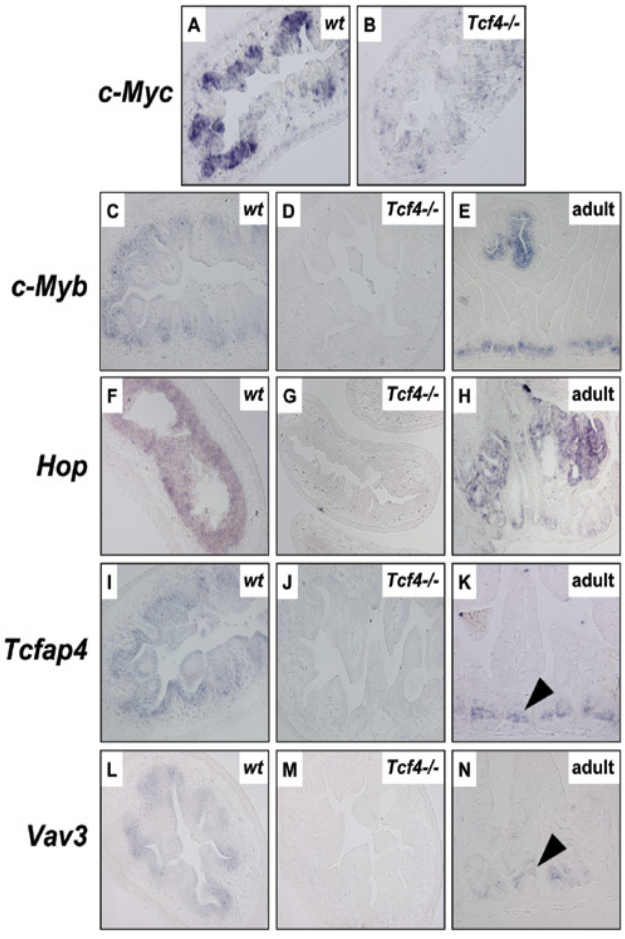


Figure 3. Downregulation of crypt progenitor markers in *Tcf4-/-* small intestines.
In situ hybridization analysis of *c-Myc* (A-B), *c-Myb* (C-D), *Hop* (E-F), *TcfAP4* (G-H) and *Vav3* (I-J) in fetal and adult small intestines.

severely abrogated in the *Tcf4-/-* intetinal epithelium. Besides these established Tcf responsive genes we uncovered novel crypt progenitor markers such as the homeodomain only protein, *Hop*; the helix-loop-helix transcription factor, *Tcfap4*; and the Rho family guanine nucleotide exchange factor, *Vav3*.

Loss of secretory lineage markers in *Tcf4-/-* small intestines

As illustrated in Figure 1, *Tcf4-/-* small intestines display a loss of enteroendocrine cells and goblet cells. Consistent with these findings, microarray profiling showed several secretory lineage markers downregulated genes in *Tcf4-/-* guts. As illustrated in Figure 4 wild-type intestines expressed, in discrete cells, abundant levels of goblet cell markers (ie. *Tff3* (282-284), *Gob4* (285), and *Gob5* (286), enteroendocrine cell markers (ie. *Cholecystokinin* and *Chromogranin B)* and the dual Paneth and goblet cell marker, *Spink4* (251). However in *Tcf4* mutant intestines the expression of these markers was severely impaired (Figure 4).

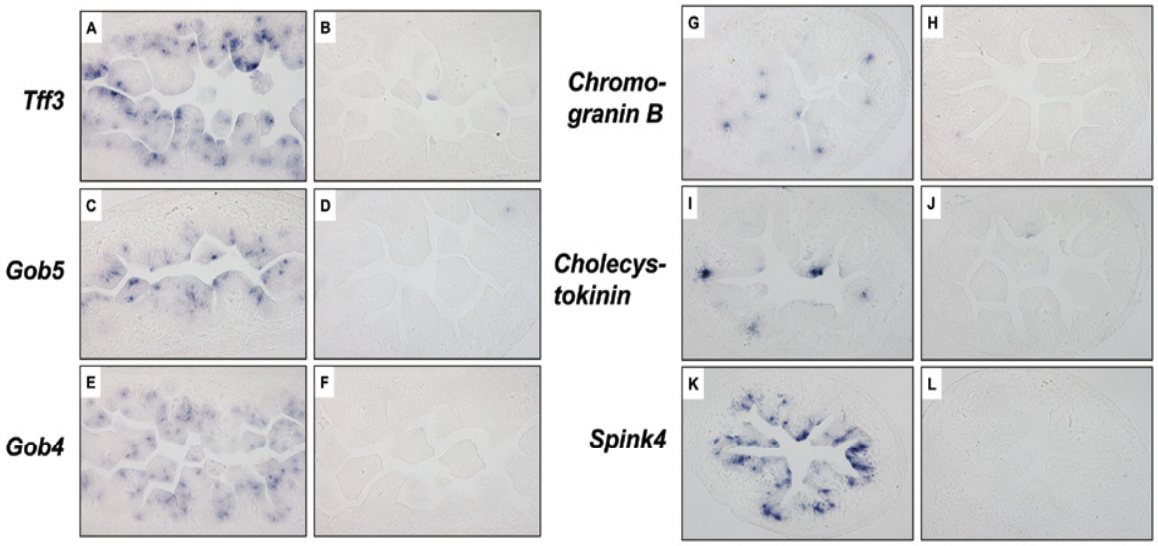


Figure 3. Downregulation of crypt progenitor markers in *Tcf4-/-* small intestines.
In situ hybridization analysis of *c-Myc* (A-B), *c-Myb* (C-D), *Hop* (E-F), *TcfAP4* (G-H) and *Vav3* (I-J) in fetal wt (A, C, E, G, I, K) and *Tcf4-/-* (B, D, F, H, J, L).

Given that removal of Tcf4 resulted in a specific blockage in the differentiation of secretory cells, we next tested whether Tcf4 controls the expression of known cell fate determinants. As was discussed earlier, all secretory cell types are derived from early progenitor cells expressing the transcription factor Math1. Moreover *Math1* deficiency results in a complete loss of secretory lineages (95). In Figure 5i and ii we found both by immunohistochemistry and RT-PCR that Math1 expression levels were reduced in *Tcf4-/-* intestines. In *Tcf4* mutants, the only remaining Math1 positive cells were restricted to the most anterior and distal regions of the small intestine in accordance with our histological analysis of *Tcf4* mutants at E18.5 in Figure 1 (data not shown). Another key regulator of secretory lineage commitment is Hes1. As a target gene of Notch signaling, Hes1 represses Math1 expression and thereby promotes commitment away from secretory lineages, towards

the enterocytic lineages (96). As shown in Figure 5i and ii, contrary to Math1, *Hes1* levels were unaffected in *Tcf4* mutant intestines. Finally to rule out the possibility that alterations in other Hes family members were responsible for the reduction in Math1 we compared the levels of three Hes genes known to be expressed in the intestine *Hes5, Hes6, Hes7 (278)*. Again however we found no differences in the expression levels of *Hes5, Hes6, Hes7* between wild-type and Tcf4 mutant intestines (Figure 5ii).

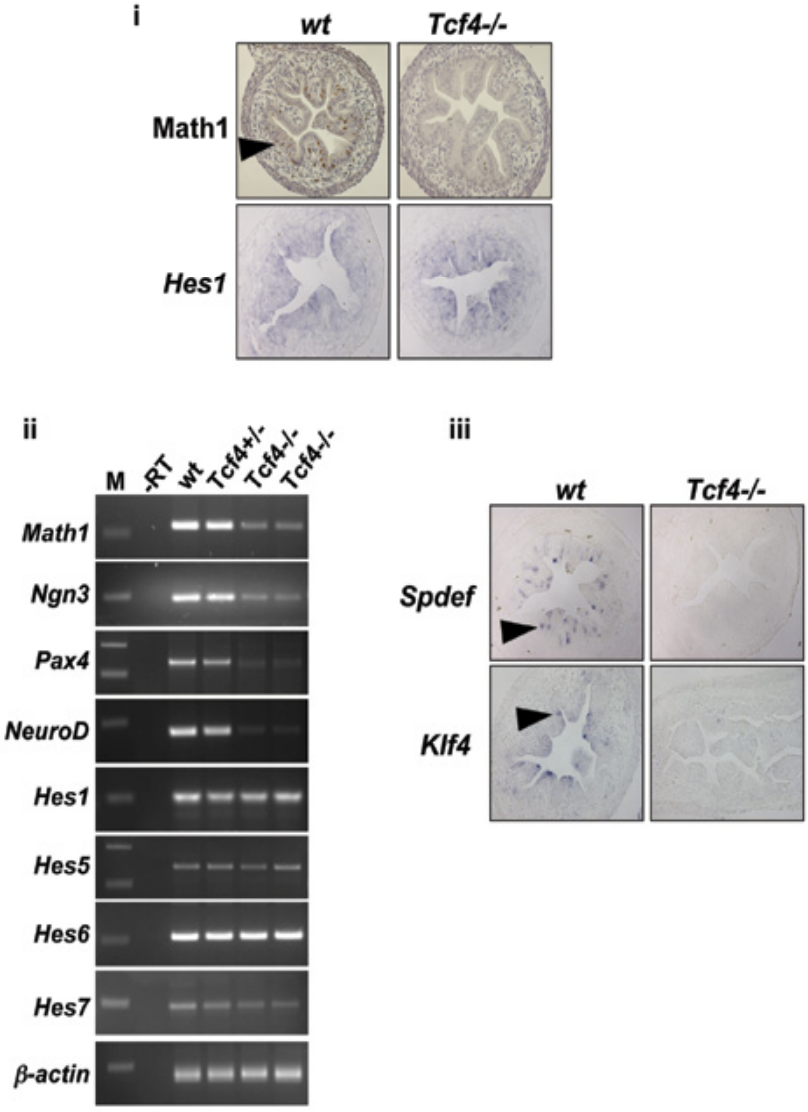
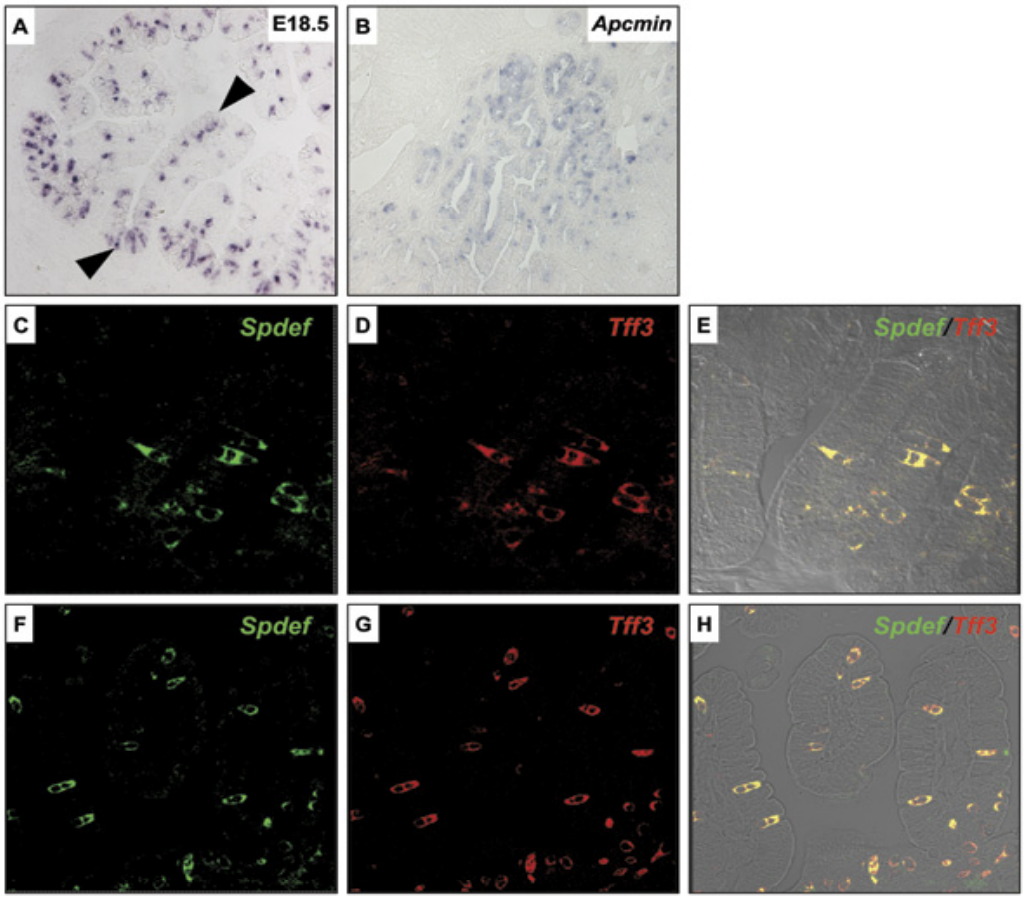


Figure 5. Downregulation of cell fate determinants in *Tcf4*$^{-/-}$ small intestines. (i) Immunohistochemistry and *in situ* hybridization analysis of Math1 and *Hes1* respectively. (ii) RT-PCR analysis on wild-type, *Tcf4*+/- and *Tcf4*-/- intestines for *Math1, Ngn3, Pax4, NeuroD, Hes1, Hes5, Hes6, Hes7 and β-actin*. (iii) *In situ* hybridization analysis on wild-type and *Tcf4*-/- intestines for the candidate goblet cell determinants *Klf4 and Spdef*. Expression of all secretory lineage cell fate determinants were downregulated in *Tcf4*-/- intestines, whereas *Hes* family members were unchanged.

Dowstream of Math1, commitment towards either enteroendocrine cells, goblet cells or Paneth cells depends on the activation status of various transcription factors. For example Ngn3 promotes enteroendocrine cell commitment, while other factors, such as NeuroD, Nkx2.2 and Pax4, regulate the differentiation of specifc enteroendocrine sublineages (99). Besides enteroendocrine cells however little is known about the factors regulating the

formation of goblet cells and Paneth cells. Loss of function studies in mice have pointed to Kruppel-like factor 4, *Klf4*, as a possible candidate specifically driving goblet cell differentiation (100). Based on co-expression studies with the goblet cell marker *Tff3*, we identified the Ets family transcription factor, *Spdef*, as another putative goblet cell regulator and a Tcf4 responsive gene (Supplementary Figure 1). In agreement with our initial finding that Tcf4 regulates Math1 expression, we observed a strong reduction in the levels of *Ngn3, Pax4, NeuroD, Klf4, and Spdef* in *Tcf4-/-* intestines (Figure 5ii and iii).



Supplementary Figure 1. Spdef is a marker of goblet cells.
(A-B) *In situ* hybridization analysis of *Spdef* in E18.5 small intestine and *APCmin* adenomas. (C-D) Double fluorescence *in situ* hybridization (FISH) analysis of the *Spdef* (green labelings, Alexa Fluoro 488) and *Tff3* (red labelings, Alexa Fluoro 546) in the adult intestine. (E-H) DIC images of an intestinal crypt (panel E) and villus (panel H) superimposed on *Spdef* and *Tff3* stainings.

Discussion

In this study we provide a detailed account of the global gene expression profiles in *Tcf4-/-* fetal intestines. Our microarray analysis was performed on whole small intestines at a developmental stage prior to the massive loss of progenitor cells observed in *Tcf4* mutants. As a result we identified three categories of Tcf4 responsive genes: upregulated enterocyte differentiation markers as well as downregulated crypt progenitor and secretory lineage markers.

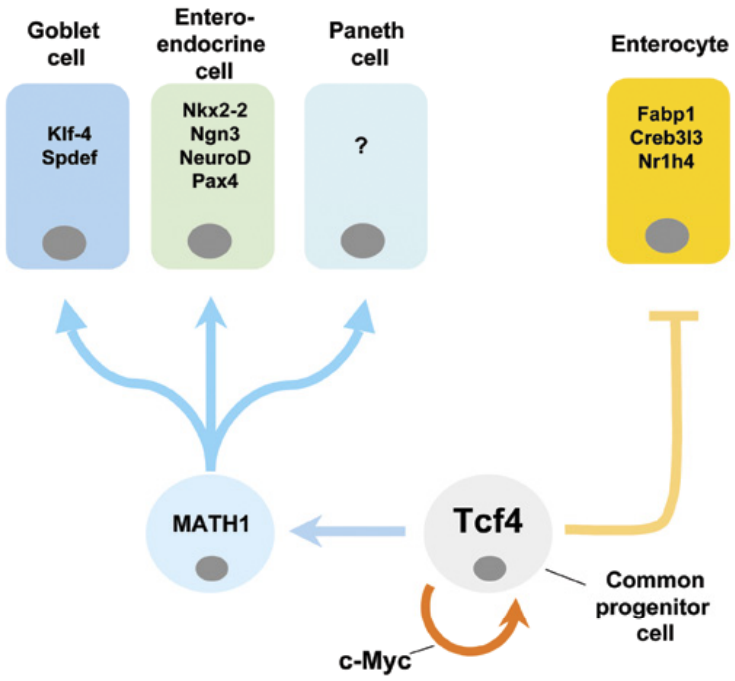


Figure 6. Model for the role of Tcf4 in intestinal epithelial cells. Our data implies a model whereby Tcf4 promotes: 1) renewal of progenitor cells in part through activation of c-Myc; 2) inhibition of enterocyte differentiation through direct or indirect transcriptional repression of markers such as *Fabp1, Creb3l3, Nr1h4,* etc.; 3) commitment towards secretory lineages through transcriptional activation of *Math1*.

As shown in Figure 2 and Table I, loss of Tcf4 resulted in a robust increase in the expression of genes associated with differentiated enterocytes. This increase was particularly evident in the distal portion of the small intestine, implying that the genetic program regulating enterocytic differentiation was prematurely turned on in Tcf4 mutants. How exactly Tcf4 regulates enterocyte lineage commitment is unclear. As shown in Figure 5, we found no evidence to suggest that effects on enterocyte differentiation were mediated by changes in the expression of Hes genes. Alternatively, the precipitated increase in enterocyte makers may reflect changes in expression levels of cell cycle regulators. Work from our laboratory has previously shown that in colon cancer cells down regulation of *c-Myc*, as a consequence of inactivation of Tcfs, deprepresses the cell cycle inhibitor p21(Cip/WAF1) (69). Moreover, the increase in p21 levels was found to be necessary and sufficient to induce cell cycle arrest and differentiation of colon cancer cells (69). Similarly in the fetal intestine, we find that *c-Myc* expression is reduced in *Tcf4* mutants, and therefore we may hypothesis that in this context, cell cycle inhibitors are consequently upregulated and possibly drive early differentiation of progenitors into enterocytes. Finally, our results may also suggest a model

whereby Tcf4 actively represses the expression of enterocytic markers, through the recruitment of general transcriptional repressors as was put forward previously (50;51).

As anticipated based on previous microarray studies, we found that a significant fraction of downregulated genes in *Tcf4-/-* intestines represented markers of crypt progenitor cells (ie. *c-Myc, Sox-9, Gpx2*). By far the best characterized of these Tcf4 target genes is *c-Myc*. Unpublished work in our laboratory has shown that conditional loss of c-Myc in the adult intestine results in reduced crypt epithelial cell proliferation and growth (Muncan V and Clevers H, manuscript submitted). Recently similar studies performed have uncovered an additional role for c-Myc in the formation of crypt structures (287). Therefore altogether these data indicate that Tcf4 maintains the self renewing capacity of epithelial progenitor cells, at least, in part by regulating *c-Myc* expression.

In addition to known crypt progenitor markers our study has identified three novel Tcf4 target genes: *Hop, Tcfap4 and Vav3*. *Hop* was discovered as a gene highly expressed in the developing heart and shown to play an essential role in regulating serum response factor mediated transcription (288;289). *Vav3* along with the other Vav family members are guanine nucleotide exchange factors involved in regulating cytoskeletal rearrangements as well as signal transduction events downstream of tyrosine kinases (290). Most studies examining Vav function have focused on lymphophocyte development and activation (291), although a recent report has shown that Vav3 is a key regulator of bone density and osteoclast function (292).The biological role of *Tcfap4* remains unknown.

The most strongly downregulated genes in *Tcf4-/-* intestines were differentiation markers of goblet cells, enteroendocrine cells and Paneth cells. This observation prompted us to examine whether early commitment of progenitor cells towards a secretory cell fate was impaired in *Tcf4* mutants. Indeed we found Math1 expression strongly reduced in the absence of Tcf4 and consequently specific cell fate determinants downstream of Math1, such as Ngn3, Pax4, NeuroD etc. were also impaired. To follow up these observations it will be interesting to test whether Math1 is a direct Tcf4 target gene.

Table I. Selected list of Tcf4 responsive genes

Genes downregulated in Tcf4-/-E15.5 small intestine

Gene Name	Accession number	Fold change	*p* value
Paneth cell markers			
cryptdin 6 (Defcr6)	NM007852	-27.29	7.78E-28
cryptdin 2 (Defcr2)	AH005399	-20.98	2.99E-25
defensin related cryptdin peptide (Defcr)	NM010031	-19.10	1.96E-27
cryptdin 3 (Defcr3)	NM007850	-16.39	2.72E-28
serine protease inhibitor, Kazal type 4 (Spink4)	NM011463	-11.74	1.47E-16
cryptdin 4 (Defcr4)	NM010039	-8.93	2.06E-19
P lysozyme structural (Lzp-s)	NM013590	-1.71	1.92E-02
Enteroendocrine cell markers			
chromogranin B (Chgb)	NM007694	-14.17	5.11E-30
secretin (Sct)	NM011328	-12.41	6.83E-30
glucagon (Gcg)	NM008100	-12.17	1.21E-08
ghrelin (Ghrl)	NM021488	-7.00	5.47E-11
cholecystokinin (Cck)	NM031161	-4.60	1.34E-07
Goblet cell markers			
chloride channel calcium activated 3 (Clca3/Gob5)	NM017474	-27.04	4.57E-31
anterior gradient 2 (Agr2/Gob4)	NM011783	-18.16	1.37E-30
serine protease inhibitor, Kazal type 4 (Spink4)	NM011463	-11.74	1.47E-16
trefoil factor 3, intestinal (Tff3)	NM011575	-10.99	3.48E-29
SAM pointed domain containing ets (Spdef)	NM013891	-3.46	4.15E-02
Kruppel-like factor 4 (Klf4)	NM010637	-1.45	6.35E-03
Crypt progenitor markers			
SRY-box containing gene 9 (Sox9)	NM011448	-7.56	5.55E-09
glutathione peroxidase 2 (Gpx2)	NM030677	-6.95	4.93E-20
vav 3 oncogene (Vav3)	NM146139	-3.40	7.62E-03
homeobox only protein (Hop)	NM175606	-2.52	5.63E-06
myelocytomatosis oncogene (Myc)	NM010849	-2.19	4.51E-04
myeloblastosis oncogene (Myb)	NM033597	-2.16	1.29E-03
inhibitor of DNA binding 1 (Idb1)	NM010495	-1.85	2.67E-06

Genes upregulated in Tcf4-/- E15.5 small intestine

Gene Name	Accession number	Fold change	*p* value
Enterocyte markers			
nuclear receptor subfamily 1, group H, member 4	NM009108	1.93	4.36E-07
fatty acid binding protein 2, intestinal (Fabp2)	NM007980	2.07	6.22E-09
fatty acid binding protein 1, liver (Fabp1)	NM017399	2.29	1.06E-10
cAMP responsive element binding protein 3-like 3	NM145365	2.49	1.21E-11
solute carrier family 26, member 1 (Slc26a1)	NM174870	2.60	1.76E-02
alpha fetoprotein (Afp)	NM007423	3.79	1.19E-18
apolipoprotein A-IV (Apoa4)	NM007468	4.64	5.15E-21
alcohol dehydrogenase 1 (class I) (Adh1)	NM007409	4.66	1.23E-21

CHAPTER 6

SUMMARIZING DISCUSSION

More than 20 years ago the founding member of the Wnt gene family, Wnt-1/Int1, was discovered as a proto-oncogene activated in mammary gland tumors by the mouse mammary tumor virus (MMTV) (293;294). Intense investigation by numerous laboratories has since clearly shown that Wnt genes and their downstream effectors play an essential role, not only during carcinogenesis, as was originally assumed, but also in a wide array of developmental processes. The major thrust of this thesis was to examine the function of Wnt signaling during early gut development, as well as intestinal organogenesis and homeostasis.

As a functional model to study Wnt signaling in this context we utilized mouse lines deficient for the T cell factors: Tcf4 and Tcf1. Tcf family members are obligatory mediators of Wnt induced transcriptional activation and consequently are considered essential components of the canonical Wnt cascade. In response to Wnt stimulation, Tcfs activate transcription by forming a complex with β-catenin, which in turn serves to further recruit general transcriptional modulators such as Brg1 and CBP (41;43;295). As with many other transcription factors, in the absence of coactivators, Tcfs may recruit general repressors and thereby actively shut off transcription of target genes (50;51). In this thesis we provide genetic evidence that the activator and possibly repressor functions of Tcfs are required for proper patterning and cytodifferentiation of the gastrointestinal tract.

In Chapter 2, we characterized the phenotype of compound *Tcf4-/-/Tcf1-/-* embryos. These mutants developed major developmental defects consisting of severe posterior truncations and early gastrointestinal malformations. The first abnormality observed was the apparent absence of hindgut, as documented by the complete loss of endodermal markers in the posterior region of *Tcf4-/-/Tcf1-/-* embryos. Although this was not addressed in Chapter 2, at least three possible explanations may account for the defects in hindgut formation. Firstly, Wnt/Tcf signals may be stimulating proliferation of posterior endodermal cells, as is known to occur at much later fetal stages in hindgut derivatives such as intestinal epithelial cells (66). Alternatively Tcf1 and Tcf4 may be promoting survival signals within endodermal cells. We believe that this scenario can be ruled out based on our observations that Tcf4/Tcf1 mutants do not show increased numbers of apoptotic cells, as evidenced by whole-mount TUNEL assays (unpublished data). Finally, besides mitotic effects, Tcf4 and Tcf1 may be regulating cell fate decision in early endodermal cells. Indeed in Chapter 2, we speculated that the ectopic neural tissue observed in the caudal regions of *Tcf4-/-/Tcf1-/-* embryos may derive from misspecified epiblast cells, which take on a neural fate instead of an endodermal fate. Similar events are thought to occur in *Lef-/-/Tcf1-/-* embryos, although in this case, presomitic mesoderm is transformed into neural tubes. It should be noted, however, that in *Tcf4-/-/Tcf1-/-* embryos the ectopic neural tissue appear as bifurcations of the neural tube and not neural tubes in place of hindgut. Consequently, the bifurcations of the neural tube may simply be a result of hyperproliferating neural cells, rather than cell fate changes in early epiblast cells. To

resolve this issue it will be necessary to determine at which time-point the ectopic neural tissue is first detected in *Tcf4-/-/Tcf1-/-* embryos.

Because *Tcf4-/-/Tcf1-/-* embryos survived until E15.5 we were able to assess the role of Tcf4 and Tcf1 during the patterning phase of the gut tube. As a consequence of the early hindgut defects, Tcf4/Tcf1 mutants displayed severely truncated intestines. Surprisingly in these mutants, we found clear evidence of gastro-intestinal transformations. In particular, the region morphologically appearing as duodenum had taken on a gastric fate. We hypothesized that this anterior transformation reflected a function for Tcfs in impeding the growth of the stomach. Interestingly, in support of this idea we find that *Tcf4-/-* stomachs display a substantial increase in the number of proliferative epithelial cells, suggesting that Tcf4 represses proliferation in stomach epithelial cells (unpublished observations). Whether this function depends on Tcf4-mediated transcriptional activation or repression remains to be resolved. Moreover if inhibition of Wnt signaling relieves growth constraints imposed upon the stomach during gut patterning, one may consider the possibility that under normal circumstances Wnt activity is effectively blocked in the prospective stomach regions of the gut tube. One possible mechanism to suppress Wnt signaling in the stomach has recently been proposed. Shivdasani and colleagues have shown that loss of the stomach specific transcription factor Barx1 prevents stomach epithelial differentiation (296). These authors went on to show that Barx1 specifies gastric fate through its ability to promote the transient expression of secreted Wnt antagonists sFRP1 and sFRP2 in the stomach mesenchyme.

In Chapter 3, we moved beyond gut development to study the localization of Wnt signaling components in the mature adult intestine. Despite the wealth of genetic evidence supporting a role for Wnt signaling in the adult intestine, very little was known regarding the expression pattern of individual Wnts and their downstream signaling components in the intestine. Previously several authors hypothesized that the Wnt ligands driving proliferation of crypt epithelial cells would be expressed in surrounding mesenchymal cells (87;88). Although we found several Wnts expressed in the villus mesenchyme none were detected in crypt regions, implying that proliferation of epithelial cells may solely be driven by autocrine Wnt signals. Otherwise, we can only conclude that crypt mesenchyhmal Wnts are expressed below the detection levels of our *in situ* hybridization protocol. The expression of Wnts and Fzs in the differentiated compartments of the crypt/villus axis, including Wnt4, Wnt5a, Wnt2b, Fz4, Fz6 implicated a role for non-canonical Wnt signaling in the intestine. As an attempt to uncover what this function might be we have since examined intestines derived from single Wnt5a and Wnt4 mutant mice. Histological analysis, however, revealed no obvious abnormalities (data not shown). Unlike the classical Wnt/β-catenin pathway, which has one major endpoint, non-canonical Wnts induce pleiotropic cellular responses. These are often categorized into two branches, including the so-called planar cell polarity (PCP) pathway and the Wnt-Ca^{++} pathway. The former stimulates cytoskeletal rearrangements through activation of the small GTPases Rho and Rac, whereas the Wnt- Ca^{++} pathway relies on the following effectors phospholipase C (PLC), calcium-calmodulin-dependent kinase 2 (CamK2) and protein kinase C (PKC) (297). Complicating matters further is the fact that

these non-canonical signaling components are also regulated by numerous other extracellular stimuli besides Wnts. Consequently a clear picture of the role for this pathway has yet to emerge.

Besides evidence for non-canonical signaling, our screen revealed a putative role for secreted Wnt antagonists, including Wif, Dkk2, Dkk3, sFRP1 and sFRP5. The most intriguing of these factors appears to be sFRP5, based on its restricted expression immediately above the Paneth cells. The precise localization of intestinal stem cells is subject to much debate. The classical view based on radiolabeling experiments suggests that in the small intestine stem cells reside on average around the fourth cell position from the crypt bottom (298). However other reports have proposed that proliferative columnar cells (in-between Paneth cells) at the crypt bases represent the bone fide stem cell compartment (299;300). Assuming sFRP5 is uniquely expressed in stem cells, we may ask what role a Wnt antagonist may play in regulating intestinal stem cells. One of the hallmarks of stem cells is their reduced mitotic rate when compared to other crypt progenitor cells (301). Therfore sFRP5 may be required to maintain stem cells in a relative quiescent state by locally blocking the stimulatory effect of Wnts on proliferation. Another important issue, assuming again this model to be correct, is how sFRP5 expression is activated specifically in stem cells. Based on the absence of sFRP5 in adenomas and most crypt progenitor cells it is unlikely that sFRP5 is regulated by Wnt signaling. In the future it will be of interest to investigate whether other signaling pathways known to affect stem cell proliferation (eg. Notch and BMP signaling) regulate sFRP5 expression. Recent work has highlighted a putative role for sFRP5 and other sFRPs in human colon cacerogenesis. The vast majority of human colon cancer cells appear to silence sFRP5 expression through methylation. Consistent with the above-mentioned model, these latest findings suggest that downregulation of sFRP5 may provide cells with a growth advantage.

In Chapter 4 and 5, we identified Wnt/Tcf4 responsive genes during fetal intestinal development. In our initial microarray experiments, we noticed that the products of Paneth cells including cryptdins were amongst the most strongly downregulated genes in *Tcf4-/-* intestines. Paneth cells are differentiated intestinal epithelial cells which unlike other differentiated lineages reside at the very bottom of the crypts of Lieberkühn, express high levels of nuclear β-catenin and secrete at least two Wnt ligands (Wnt3 and Wnt9b). To demonstrate that Paneth cells depend on Wnt signaling to drive part of their genetic program we utilized Fz5 conditional mutant mice. As a result of defective expression of the Tcf target gene, EphB3, Fz5 deficient Paneth cells did not home the bottom of the crypts but rather scattered throughout the epithelial lining. In these mis-positioned Paneth cells cryptdin expression was abrogated. This result indicated that Wnt signaling controls proliferation of progenitors but also drives maturation of epithelial cells by regulating a post-mitotic genetic program.

In Chapter 5, a more exhaustive analysis of Tcf4 responsive genes allowed us to further propose that Wnt/Tcf4 signals are involved in commitment of progenitor cells towards secretory lineages and that Tcf4 negatively regulates enterocyte differentiation. Indeed in

Tcf4-/- intestines we found a reduction in secretory cell fate determinants such as Math1 as well as Ngn3, NeuroD, Pax4 etc. and upregulation of enterocyte markers. Importantly these Tcf4-mediated effects were independent of Hes1, the well established Math1 repressor and early enterocyte determinant.

A number of questions arise from these observations. Firstly, how does Tcf4 regulate Math1 expression? To answer this question we are now looking at the possibility that Tcf4 directly activates transcription of the Math1 gene. Obviously we must also consider the possibility that Tcf4 regulates Math1 via an indirect route. Regardless of the mechanism it is clear that Math1 expression is not entirely blocked following loss of Tcf4. Math1 positive cells were frequently detected near the gastro-duodenal junction and the very distal portion of *Tcf4-/-* intestines, albeit at reduced levels when compared to wild-type intestines (data not shown). These positional effects suggest that other factors can compensate for or take over Tcf4 function, depending on the compartment of the intestinal tract one is examining. Further supporting this view is our observation that at E18.5 in the proximal portion of the colon, loss of Tcf4 does not result in any changes in goblet cells numbers, despite the absence of proliferative cells (data not shown). It is unclear from the current reading of the literature whether these positional effects also occur in other mouse models displaying defective Wnt signaling in the intestine (ie *β-catenin-/-* and DkkTg mice) (65;68).

On a related issue we may also question how Tcf4 negatively regulates the expression of enterocyte markers. . In Chapter 4, we demonstrated that Tcf4 promotes Paneth cell differentiation by directly activating cryptdin gene expression. In a similar manner we may propose that Tcf4 inhibits enterocyte maturation by directly repressing markers such as Fabp1, Creb3l3 and Nr1h4 etc. As with Math1, chromatin immunoprecipitation experiments will need to be performed to verify this hypothesis. As an alternative explanation, in Chapter 5 we discussed an attractive model initially proposed by van de Wetering et al. (69), whereby Tcf4 indirectly regulates enterocyte differentiation by controlling c-Myc, and in turn, p21WAF/CIP1 levels. Loss and gain of function experiments suggest that in progenitor cells p21 is repressed by c-Myc, and as c-Myc levels drop, p21 is derepressed and can stimulate differentiation of progenitors. To test this hypothesis we are now examining whether expression of p21 precedes or coincides with the increase in expression of enterocyte markers seen in *Tcf4-/-* intestines. If so, this observation would provide indirect evidence supporting the above model. In this context it would also be interesting to verify whether the ectopic increase of enterocytic markers as a result of the inactivation of Tcf4 is blocked in a p21 deficient background. Besides p21 we shall also examine other cell cycle inhibitors, such as p27KIP1 and p57KIP2, both of which have been linked to differentiation processes in intestinal epithelial cells (302;303).

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Abbreviations

ISH: *in situ* hybridisation;
Wnt: Wingless and int-1;
Fz: Frizzled;
LRP: low-density lipoprotein (LDL)-receptor-related protein;
sFRP: secreted frizzled-related protein;
WIF: Wnt-interacting factor;
Dkk: Dickkopf;
TCF: T-cell factor;
LEF: lymphoid enhancer factor
APC: Adenomatous polyposis coli
CRC: Colorectal cancer

Summary

The Wnt pathway controls diverse biological processes during embryonic development. In the adult, Wnts maintain the balance between cell division and cell specialisation in tissues such as the hemapoetic system, skin, and the intestine. Genetic modifications which activate the Wnt pathway are also closely linked to unrestricted cell growth and malignancy. In this thesis we focused on the specific role of the Wnt pathway during normal intestinal development and homeostasis. To this end, we utilized so-called knockout mice which harbour mutations in the essential componenents of the pathway and studied the molecular consequences of these mutations by *in situ* hybridization, a technique which allows one to visualize the expression of gene products in tissues.

In Chapter 2 we studied the consequences of deleting two Wnt effector proteins Tcf4 and Tcf1 compound null embryos. Mouse lacking the gene products of both *Tcf1* and *Tcf4* showed severe caudal truncations of the body, as well as duplications of the neural tube. Unlike other mutations affecting Wnt signaling, paraxial mesoderm formation was not impaired and early caudal markers, such as *T*, were unaffected. Analysis of endodermal markers uncovered early and specific defects in hindgut expansion and later an anterior transformation of the gastro-intestinal tract. Our results reveal a novel role for Wnt signalling in early gut morphogenesis and suggest that specific Wnt-driven patterning events are determined by the unique tissue distribution of *Tcf/Lef* family members.

In Chapter 3 we performed a large scale *in situ* hybridisation screen to examine the expression pattern of all Wnts, Fzs, LRPs, Wnt antagonists and TCFs in the murine small intestine, colon and adenomas. Our analysis revealed high expression of several signaling components (including *Wnt-3, Fz-7,*) in crypt epithelial cells. We also detected gene products such as *Wnt-2 and Fz-6* in differentiated epithelial and/or mesenchymal cells of the small intestine and colon. Finally, several factors (*TCF-1, Dkk-3*) displayed differential expression in normal versus neoplastic tissue. This study predicted a much more complex role for Wnt signaling in gut development and homeostasis than was previously anticipated.

In Chapter 4 we found that inactivation of Tcf4 in the embryonic intestine and conditional deletion of the Wnt receptor Frizzled-5 in the adult intestine abrogated a specific Paneth cell genetic program. Paneth cells secrete microbicidal peptides, such as cryptdins, important in fighting bacterial infections. Moreover these Paneth cells reside at the very bottom of the intestinal crypts. In Tcf4 and Fz-5 mutant mice we observed a defect in the production of Paneth cell gene products and a scattering of Paneth cells throughout the crypt-villus axis. Conversely, adenomas in APC mutant mice and colorectal cancers in man inappropriately expressed these Paneth cell genes, providing additional support that the expression of these genes is driven by active Wnt signals. Furthermore, these observations implied that Wnt signals in the crypt can separately drive a stem-cell/progenitor gene program and a Paneth cell maturation program.

Finally in Chapter 5 we studied in more detail the defects associated with loss of Tcf4 in the intestine. Deletion of Tcf4 results in neonatal death and a complete loss of proliferative stem cells in the intestine. By monitoring the gene products of specialized cell-types we found that absorptive cell markers (ie. *Fabp1, Creb3l3, Nr1h4,* etc.) were upregulated in Tcf4 knockout embryos. Concomitantly, loss of Tcf4 resulted in specific downregulation of secretory lineage markers (ie. *Tff3, Chromogranin B, and Spink4,* etc.) and crypt progenitor markers (ie. *c-Myc, c-Myb, TcfAP4,* etc). Further analysis suggested that Tcf4 promotes early commitment of secretory lineages through activation of the basic helix-loop-helix transcription factor, Math1. Moreover we found that Tcf4-mediated effects on cell fate were independent of any changes in the expression of Hes family members. Finally our results imply a model whereby Tcf4 coordinates renewal of progenitor cells, repression of enterocyte differentiation and commitment towards secretory lineages via Math1.

Samenvatting

(Marc Vooijs PhD)

De Wnt signalering keten dirigeert een verscheidenheid aan biologische processen gedurende embryonale ontwikkeling in zoogdieren. Ook in volwassenen, reguleren Wnt eiwitten de balans (homeostase) tussen celdeling (proliferatie) en celspecialisatie (differentiatie) in bijvoorbeeld de huid, de darmen en de vorming van bloedcellen. Verstoring van deze balans door bijvoorbeeld genetische veranderingen in het DNA leidt tot ongeremde groei van cellen en uiteindelijk tot kanker. In dit proefschrift hebben we ons gericht op rol die Wnt eiwitten hebben op darmontwikkeling en homeostase. Door gebruik te maken van genetische gemodificeerde muizenstammen die specifieke genproducten niet meer kunnen maken (knockout muis) kunnen we onderzoeken wat de functie van dit eiwit is op de normale darmontwikkeling.

In hoofdstuk 2 beschrijven we de consequenties van de afwezigheid van twee belangrijke componenten van de Wnt signaaltransductieroute; Tcf-1 en Tcf-4 op (darm)ontwikkeling in muizen. De afwezigheid van de Tcf-1 en Tcf-4 eiwitten in muizen leidt tot een ernstige verstoring van de embryonale ontwikkeling, in het bijzonder tot een complete afwezigheid van de achterpoten en staart en een verdubbeling van neurale buis structuren. In de vroege darmontwikkeling leidt de afwezigheid van Tcf-1 en Tcf-4 tot een defect in de uitgroei van het meest naar achter gelegen structuren. Een gedetailleerde analyse van markers voor darm cel specificatie toonde aan dat er een defect in cel identiteit was opgetreden zodanig dat de normaliter meer naar achter gelegen structuren de identiteit aannamen van meer naar voren gelegen darm (anteriore transformatie).
Deze resultaten brengen een nieuwe rol voor Wnt eiwitten aan het licht in de aanleg en vorming van de eerste darmstructuren die gekaracteriseerd worden door de aanwezigheid van een uniek patroon van Tcf-1 en Tcf-4 gen expressie.

In hoofdstuk 3 beschrijven we een gen-expressie analyse van de meeste componenten van de Wnt signalering cascade waaronder alle tot nu toe bekende Wnt eiwitten, Frizzeld's (Fz) en LRP's de receptor en co-receptor voor Wnt eiwitten respectievelijk, natuurlijke remmers van Wnt eiwitten (Dickkopff) en Tcf factoren in de darmen van normale muizen en die van muizen met darm kanker. Uit deze analyse bleek dat sommige Wnt's en hun Fz receptor alleen in de ongespecialiseerde darm epitheel cellen tot expressie kwamen (b.v. Wnt-3 en Fz7) terwijl anderen in het meer gedifferentieerde darm epitheel (Wnt2b, Fz6). Tevens bleken sommige Wnt's uitsluitend in het, naast het epitheel liggende, steun weefsel tot expressie te komen suggererend dat er een intieme communicatie is tussen deze twee cel typen dat normale cellulaire homeostase reguleerd. Bovendien vonden we verschillen in expressie van verscheidene componenten tussen de normale darm en darm tumoren (Tcf-1, Dickopff). Deze informatie leidt tot nieuwe inzichten in de complexiteit van de mogelijke interacties tussen componenten van de Wnt signaleringsroute met het omliggende darmweefsel en verstoring daarvan tijdens darm kanker ontwikkeling.

In hoofdstuk 4 beschrijven we de consequenties van de afwezigheid van Frizzled-5 (Fz-5) op darmontwikkeling en functie in volwassen muizen. In de normale volwassen darm vindt een continue proces plaats van cel vernieuwing dat er voor zorg draagt dat uitgerijpte gespecialiseerde darm cellen na ongeveer een week vervangen worden door nieuwe. Dit proces wordt gereguleerd door stam cellen waarvan na deling de nakomelingen nieuwe cel identiteiten aannemen. Het aannemen van deze nieuwe identiteit en functie gaat gepaard met een opwaartse of neerwaartse verplaatsing van deze cellen in de darm structuren. Deling van darm stam cellen staat onder controle van de Wnt signaleringsketen. Muizen die het Tcf-4 eiwit missen produceren geen darm stam cellen. Uitschakeling van Fz-5 in de darmen van muizen had geen effect op de functie van de stam cellen maar op de cel specialisatie van de cellen van Paneth. Deze cellen dragen ondermeer zorg voor bescherming tegen bacterieen in de darm. Normaliter bewegen de cellen van Paneth zich in een neerwaartse beweging na deling van de darm stam cel, echter in de afwezigheid van Fz5 bleek er een defect in de sortering van Paneth cellen. We vonden Paneth cellen terug in alle structuren van de darm waar ze normaliter niet thuis horen. Bovendien vonden we dat darm kanker cellen gen-expressie patronen vertoonden van de gespecialiseerde Paneth cellen. Hiermee toonden we voor het eerst aan dat de Wnt signaleringsketen niet alleen een belangrijke rol heeft in de regulatie van stam cel deling maar ook een gerichte functie heeft in de uitrijping en sortering van de cellen van Paneth.

In hoofdstuk 5 hebben we ons gericht op de darm defecten van Tcf-4 knockout muizen. Afwezigheid van Tcf-4 in muizen is niet levensvatbaar maar leidt wel tot een normale ontwikkeling van darm structuren. Deze darmen missen echter stam cellen die na de geboorte noodzakelijk zijn voor de continue cel vernieuwing die dan plaatsvindt.
Afwezigheid van Tcf-4 resulteert in een defect waardoor veel cellen de moleculaire identiteit van asorptieve cellen aannamen gepaard met een verlies is van stam cel identiteit. Uit onze analyse bleek gedurende normale darm ontwikkeling aansturing van het BHLH eiwit Math1 door Tcf-4 essentieel bleek te zijn voor normale uitrijping darmepitheel. Omdat de activiteit van het Math1 ook onder invloed staat van de Notch signaleringroute onderzochten we of defecten van Tcf-4 op Math1 afhankelijk waren van Notch signalering, dit bleek echter niet zo te zijn. Samenvattend blijkt uit onze analyse dat Tcf-4 de balans tussen stam cel deling en differentiatie reguleert via Math1.

Dankwoord

As I've never been a long-winded individual I prefer to keep this short and sweat. Like most in this crazy business, I've had my difficult moments filled with failed experiments and false hopes. But when looking back on my time in the Lab, I can honestly say that it's been a tremendous learning experience and lots of fun. All of this thanks to the support, insight, and kindness of so many people.

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I wish you all the very best in all your future endeavours!!

About the author

The author of this thesis was born in Montréal, Québec, Canada on May 8th 1973. Following a normal but happy childhood in Charlesbourg, a "banlieue" of Quebec City, the author attended his first biology lessons at St. Lawrence College where he later developed an affinity for dissecting sharks and other organisms. In 1993 he entered the biology program of Université Laval and in 1994 he successfully performed his first minipreps under the supervision of Dr. Moss at the Centre de Recherche de l'Hôtel-Dieu de Québec. After receiving his Bachelor of Science degree, he pursued a Master of Science degree at McGill University in the laboratory of Dr. Veillette and later in 1999 he heard a calling to move to the Netherlands to start a PhD in the laboratory of Dr. Clevers. In 2000/2003 he played an instrumental role in the promotion of URC to the 1st division of the Nederlands Rugby Bond. The next year he suffered a major injury putting on hold his amateur rugby career. In 2005, he was married to Agnieszka Masztalerz in Proszowa, Poland. Following his PhD defense on January 30th 2006 he is expecting to become a father.