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WNT signaling in the normal intestine and colorectal cancer

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1. ABSTRACT

The intestinal epithelium is a self-renewing tissue that represents a unique model for studying interconnected cellular processes such as proliferation, differentiation, cell migration and carcinogenesis. This review covers work from the past decade and highlights the importance of the canonical Wnt pathway in regulating multiple aspects of intestinal homeostasis. Numerous *in vivo* studies combined with gene profiling experiments have shown that Wnt signaling promotes maintenance of epithelial stem cells and early progenitors by driving transcription of genes associated with proliferation. These studies also revealed strong similarities between the genetic program initiated by Wnt signals in normal crypt progenitors and in colorectal cancer cells. More recently it has become apparent that Wnts do not act alone but rather cooperate with Notch signals in maintaining progenitor cell populations. Processes associated with differentiated epithelial cells also appear to be regulated by Wnt signals. For instance, Paneth cells employ active Wnt signals for terminal differentiation. Moreover, through transcriptional regulation of members of the Eph and Ephrin families, Wnt signaling promotes compartmentalization of epithelial cells along the crypt-villus axis. The Eph/Ephrin system also operates to limit progression of colorectal cancer beyond the early stages.

2. EMBRYONIC ORIGIN OF THE INTESTINE

The intestinal tract constitutes a tube composed of three tissue layers. The outer and middle layers are of mesodermal origin and consist of smooth muscle cells, responsible for peristalsis, and connective stromal tissue, respectively. The inner, endoderm-derived, layer contains epithelial cells responsible for food processing and uptake of nutrients.

In mice, intestinal development starts at embryonic day 6.0 (E6.0) when epiblast cells, committed to form definitive endoderm, travel through the primitive streak to populate the anterior end of the embryo. Endodermal cells leaving at later stages colonize more posterior regions. From E7.5 to 9.5, the endodermal layer underlying 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. The primitive gut is composed of a uniform layer of cuboidal endodermal cells covered by splanchnic mesoderm, which subsequently is reshaped along the anterior–posterior (A-P) axis. During this phase (E9.5-E14.5) the intestine, along with the other organs of the digestive tract become morphologically distinguishable. Following A-P patterning, the intestinal tract differentiates

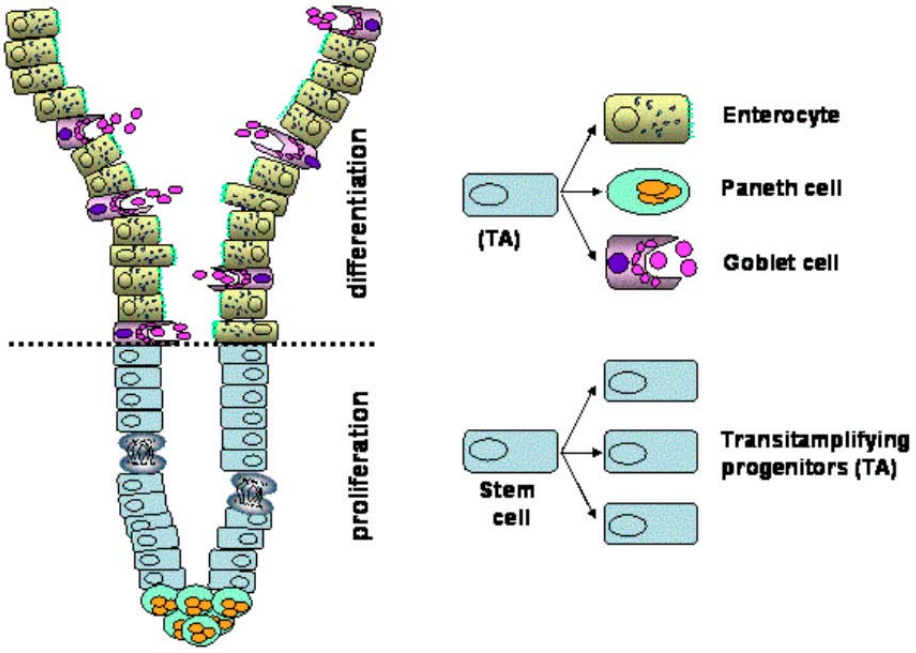

Figure 1. Schematic representation of the architecture of the intestinal epithelium. The epithelium (mucosa) of the small intestine is organized into morphologically and functionally distinct compartments. The crypt of Lieberkühn represents the proliferative compartment of the intestinal epithelium. Crypts are present in both the small intestine and colon but are larger in the colon. No villi are present in the colon. The differentiated compartment consists of specialized cells, adapted for food processing, and is found on the villi in the small intestine whereas the colon has a flat layer of similar specialized epithelial cells instead. Crypts harbor slowly dividing multipotent stem cells. The immediate descendents of these stem cells in the crypt undergo more rapid proliferation, and are referred to as transit amplifying (TA) cells. During migration of these TA-derived daughter cells to the villus surface, they differentiate into the four mature epithelial lineages. Absorptive enterocytes produce enzymes that help to digest sugars and proteins. The other three lineages are of a secretory type: Goblet cells produce mucus, thereby providing protection to the epithelium against shear stress and chemical damage. Enteroendocrine cells secrete a great variety of hormones such as Secretin, Serotonin, Substance P and Somatostatin. Paneth cells differentiate while moving to their functional location, the bottom of the crypt. They are specialized in the secretion of antimicrobial peptides termed defensins, or cryptdins, and enzymes such as lysozyme and phospholipase A2.

in a radial direction, giving rise to various mesenchymal components such as smooth muscle and stromal cells, as well as columnar epithelial cells. It is believed that these regional patterning events are driven by extensive cross-talk between endodermal and mesodermal layers through the action of soluble signaling factors (1). By E16.5, the epithelial lining becomes organized into finger-shaped luminal protrusions, termed villi, and intervillus regions consisting of highly proliferative progenitor cells. During postnatal development the intervillus pockets give rise to flask-shaped crypts, the eventual home of intestinal stem cells (reviewed in 2, 3). Similar events take place in the colon.

3. THE EPITHELIAL LINING OF THE ADULT SMALL INTESTINE AND COLON

The epithelium (mucosa) of the mammalian small intestine and colon is organized into morphologically and functionally distinct compartments (Figure 1). The crypt of Lieberkühn represents the proliferative compartment of the intestinal epithelium. Crypts are present in both the small intestine and colon but are larger in the colon. No villi are present in the colon. The differentiated compartment consists of specialized cells, adapted to various aspects of food processing, and is found on the villi in the small intestine whereas the colon has a flat layer of similar specialized epithelial cells. While crypts are monoclonal, each villus receives cells originating from different crypts and is therefore polyclonal. The lifecycle of most of the individual epithelial cells within the intestinal mucosa spans less than a week. In the mouse, stem cells in each crypt ultimately generate some 200 cells per day. These newly formed cells move upwards along the villus and differentiate. Finally, they enter apoptosis and are exfoliated from the apex of the villi in the small intestine or from the flat surface epithelium in the colon. On average, the entire intestinal epithelium is renewed every 3 to 5 days for mouse and man, respectively.

Most of our current understanding of the dynamics of this process of continuous self-renewal is based on the classical studies performed by Potten and colleagues using the adult mouse small intestine as a model system (4). These studies have shown that crypts, of which there are about one million in an adult mouse small

intestine, harbor slowly dividing multipotent stem cells (cell cycle period of 24 h) that reside in the lower third part. The immediate descendents of these stem cells occupy the middle third of the crypt and are referred to as transit amplifying (TA) cells. This TA population undergoes more rapid proliferation (cell cycle period of 12 h). During migration of these TA-derived daughter cells to the villus surface, they differentiate into the four mature epithelial lineages. Absorptive enterocytes produce enzymes that help to digest sugars and proteins. They are the most abundant cells in the epithelium and their specialization in absorption of digested material is evident by the presence of apical microvilli. The other three lineages are of a secretory type: Goblet cells (5) are most abundant in the small intestine and produce mucus thereby providing protection to the epithelium against shear stress and chemical damage. Enteroendocrine cells (6) secrete a great variety of hormones such as secretin, serotonin, substance P and somatostatin. Paneth cells (7) are specialized in the secretion of antimicrobial peptides termed defensins, or cryptdins, and enzymes such as lysozyme and phospholipase A2.

As described above, maturation of progenitor cells coincides with upward migration of cells in coherent bands along the crypt villus axis. By contrast, stem cells maintain their position in the lower part of the crypt. Paneth cells also escape this flow. While it is believed that Paneth cells are generated in the upper third part of the crypt, they migrate in the opposite direction towards the bottom of the crypt. Another characteristic of Paneth cells, that discriminates them from the other differentiated cells types in the mucosa (8), is their extended life span of 20 days.

4. SECRETED WNT PROTEINS REPRESENT THE MAIN DRIVING FORCE FOR THE ACTIVITY OF INTESTINAL STEM CELLS

4.1. Properties of intestinal stem cells

The crypt-to-villus hierarchical migratory pattern of cell proliferation and differentiation is well established and it is therefore assumed that the multipotent stem cells that give rise to the four differentiated cell lineages are located at the origin of this system, the intestinal crypt. There is a paucity of specific markers for these stem cells. Consequently, they are typically identified by their ability to retain radiolabelled thymidine for extended periods of time. Incorporation of radioactive label can be achieved at the times when new stem cells and new template DNA strands are being produced. This will occur either during gut development or following injury. Unlike all other cells, the stem cells retain abundant DNA label (9). These early experiments revealed the position of the putative stem cells at the base of the colonic crypts and approximately at cell position +4 in the small intestine. Retention of the radiolabeled thymidine is consistent with selective segregation of the newly synthesized DNA strands in these cells. Similar recent experiments using this approach, in which [^{3}H]-thymidine and BrdU were injected successively into developing mice or adult mice after irradiation, confirmed this hypothesis (10, 11). Based on these experiments, it has been proposed that stem cells in the intestine segregate their chromatids asymmetrically. The thymidine label is retained in the ‘immortal’ DNA strand while the BrdU label is incorporated in the strands donated to the immediate descendants, the TA cells. The ability of stem cells to continuously reuse the same DNA strand as template is postulated to protect them against DNA-replication-induced errors because these will be passed into the differentiating, short-lived daughter cells.

The described asymmetric segregation of DNA strands implies that some properties of stem cells may be cell autonomous. This certainly does not apply to all stem cell and/or TA cell properties. Hermiston et al. (12) found, by forced over expression of Fabp1 (Fatty acid binding protein) promoter-driven E-Cadherin in mouse intestinal epithelium, that proliferation is suppressed as well as cell movement up the villus. The slowed migration is not accompanied by a change in distribution of terminal differentiation markers along the crypt-villus axis suggesting cell nonautonomous differentiation

The retention of the stem cell properties in one of the daughter cells, which must occur in each cell division following DNA replication, also may not be a completely intrinsic feature of the stem cell. Several experimental approaches seem to indicate that stem cells not exclusively perform these asymmetric divisions. A more stochastic pattern of division, that also occasionally includes symmetric divisions in which either two stem cells or two differentiating daughter cells are produced, would argue in favor of a niche regulated stem cell behavior. Both analysis of the transition from juvenile to adult epithelium in mouse small intestine aggregation chimeras (13) and the study of CpG methylation tags in three non expressed loci in human colon propose a ‘niche succession’ model of stem cell dynamics within the crypt (14). This model suggests that stochastic extinction of stem cell lines by symmetric division results in a ‘bottleneck’ effect wherein all cells within the crypt are related to a single stem cell descendant. The estimated time taken for this bottleneck to develop was 8.2 years in normal human colon. Symmetric divisions in which clonal expansion yields two stem cells must be instrumental during regeneration of damaged tissue, in response to irradiation and during the postnatal period in which a massive increase in the number of crypts occurs in both the small intestine and colon.

Production of all epithelial lineages is another characteristic that defines stem cells. A large body of evidence, obtained from experiments with aggregation chimaeras and mosaic individuals, supports this idea known as the ‘Unitarian’ hypothesis (15). An example of the use of chimaeras can be found in DBA staining of cells. This lectin binds to cells derived from B6 mice but not to SWR-derived cells. DBA staining thus readily segregates cells from the two different strains. Neonatal chimeras will have mixed, polyclonal crypts for the first two weeks of life. In older mice, only DBA positive or negative crypts can be identified (16). A very convincing example of the exploitation of cellular mosaicism to support the Unitarian hypothesis came from Novelli et al. (17) who studied the colon of a rare XO/XY patient who required colectomy.

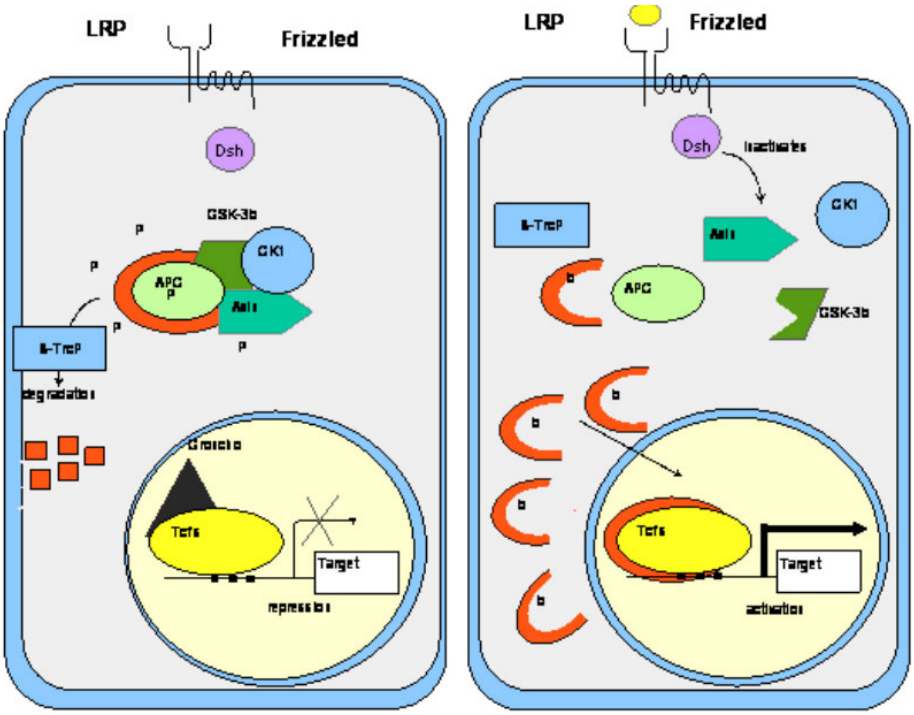

Figure 2. Simplified scheme of the canonical Wnt pathway. Left. In the absence of Wnt ligand, beta-catenin is sequestered in a multiprotein degradation complex containing the scaffold protein Axin, the tumor suppressor gene product APC, as well as the kinases CK1 and GSK3beta, among others. Upon sequential phosphorylation, beta-catenin is ubiquititylated by the beta-TrcP-E3ligase complex and subsequently degraded by the proteasome machinery. Right. Wnt ligand associates with Frizzled/LRP co-receptors. By an as yet unknown mechanism, Dishevelled now inactivates the destruction complex. beta-catenin accumulates in the cytoplasm and subsequently travels to the nucleus. Wnt target gene transcription is initiated following binding of beta-catenin to TCF/Lef transcription factors. Transcription is mediated trough the C-terminal transactivation domain in beta-catenin. There are indications that also BCL9 and Pygopus proteins are required.

Detection of the Y chromosome revealed that the normal crypts of this FAP patient were almost entirely comprised of either Y-positive or Y-negative cells. Immunostaining for the enteroendocrine cells confirmed that they shared the karyotype of the other cells in the crypt. Both these examples provide convincing evidence that all the cells in crypt are derived from the same stem cell.

It is clear that the maintenance of a healthy stem cell population in the intestine is of great importance. We have mentioned several findings indicating that regulation of stem cell activity is unlikely to be completely cell autonomous. Indeed, evidence is now accumulating that stem cells receive instructions from cells in the surrounding tissue. The first strong molecular evidence for such a niche-regulated intestinal stem cell biology came from gene targeting of the Wnt signaling effector Tcf4 (18). Mice lacking this transcription factor, although able to sense the soluble Wnt signals transmitted by the surrounding cells, are unable to respond to it. As a result these mice are hampered in generating or maintaining their intestinal stem cell compartment. Wnt signaling is now regarded as the driving force in the self-renewal of the epithelium. Before further elaborating on the evidence supporting this we will first provide a detailed description of this signaling cascade.

4.2. The Wnt cascade

The discovery that the mouse mammary oncogene int-1 appeared homologous to the Drosophila segment polarity gene Wingless (19) laid the keystone for a signaling pathway now referred to as the canonical Wnt pathway (Figure 2). This signaling cascade was subsequently found to be conserved in all metazoans where it proved to be involved in many developmental decisions (20). The human and mouse genome each harbor approximately 19 different Wnt genes that encode secreted cysteine-rich glycoproteins. To be biologically active, Wnt proteins are dependent on palmitoylation (lipid modification) of a conserved cysteine residue (21). This posttranslational modification is possibly mediated by an endoplasmic reticulum multispanning transmembrane protein, homologous to the Drosophila segment polarity gene product Porcupine (22). Signaling is initiated when secreted Wnt ligands engage their cognate receptor complexes in membranes of Wnt-responsive cells. These complexes consist of a member of the Frizzled (FZD) family of the seven span transmenbrane serpentine receptors (23) and a single-span receptor belonging to the low-density lipoprotein receptor-related protein (LRP) family (24), the vertebrate LRP5 or LRP6 and the Drosophila arrow gene product (25). The requirement of both these receptors for canonical Wnt signaling was

revealed by loss of function studies in Drosophila. Deletion of the gene encoding for arrow or combined loss of the two FZD receptors (26) phenotypically copied wingless mutation. Some Wnts may also bind to FZD receptors in the absence of LRP co-receptors to initiate an alternative response referred to as planar cell polarity pathway or non-canonical Wnt signaling (27). There are no data available supporting a role for this type of signaling in intestinal development or function.

The interaction of Wnts and their receptors ultimately regulates the stability of the multifunctional protein beta-catenin, the key player in this pathway. In cells quiescent with respect to Wnt signaling, cytoplasmic beta-catenin can only be found anchored to the cell membrane where it participates in E-cadherin mediated intercellular adhesion through the formation of adherens junctions (reviewed in 28). In contrast, initiation of signaling, leads to accumulation of unbound beta-catenin in the cytoplasm and nucleus. A dedicated destruction complex tightly regulates the size of the cytoplasmic pool of signaling-competent beta-catenin. When Wnt receptors are not engaged, two scaffolding proteins in this destruction complex, the tumor suppressors adenomatous polyposis coli (APC) and axin, bind freshly produced beta-catenin. This allows casein kinase 1 (CKI) to phosphorylate the N-terminus of beta-catenin at Ser 45 (29). This primary phosphorylation event promotes additional phosphorylations at conserved Ser and Thr residues, N terminal to Ser 45, that are performed by glycogen synthase kinase 3 beta (GSK3beta)(30). The resulting phosphorylation motif earmarks beta-catenin as a substrate for ubiquitylation by the F-box containing E3 ligase beta-TrCP and subsequent proteasomal degradation (31).

In contrast, FZD/LRP occupancy by Wnts blocks the enzymatic activity of the destruction complex by an as yet incompletely resolved mechanism. Recent evidence indicates that axin, an essential component of the destruction complex, is recruited to the membrane embedded LRP co-receptor upon receptor triggering. Exchange of axin between destruction complexes and binding sites in the cytoplasmic tail of LRP/Arrow would reduce the activity of the destruction complex, and allow for beta-catenin-mediated signaling, by withdrawal of one of its essential scaffolds. In the intracellular domains of LRP5/6/Arrow the axin docking site consists of a, five times reiterated, phosphorylated PPPsP motif (32). The Wnt induced kinase responsible for mediating this event awaits identification, as does the regulatory phosphatase. This model does not explain how receptor occupancy leads to release of axin from the destruction complex. It is also unclear whether sufficient LRP-associated docking sites are available to significantly deplete destruction complexes of their axin component. Prolonged receptor occupancy eventually results in a decrease in the level of axin raising a second opportunity for reduced destruction complex activity (33). The latter mechanism, however, cannot be responsible for canonical Wnt signaling in general since signaling occurs before an obvious decline in the level of axin can be detected (34). Moreover, receptor occupancy also induces expression of another negative regulator of the pathway, the axin homolog conductin (also referred to as axin 2) (35). This negative feedback mechanism would replenish the function of the lost axin since conductin can functionally replace axin1 in the destruction complex (36).

Genetic evidence has revealed that the adaptor protein Dishevelled (Dsh) is, in addition, essential for signal transduction (37). Biochemical evidence shows that Dsh binds to the cytoplasmic tail of FZD (38) and that removal of the docking site for Dsh in the FZD receptor blocks canonical Wnt signaling (39). The observation that Dsh seems required for the recruitment of axin to LRP/Arrow (40) seems in agreement with its ability to bind to both FZD and axin (41). Several roles can be considered for the contribution of Dsh in this model. It may for instance form a link between receptor engagement and release of axin from the destruction complex. Its adaptor function may help to stabilize axin binding to LRP/FZD or participate in the phosphorylation of LRP. Dsh may also function in a different model. In Wnt signaling-quiescent cells Dsh may be bound to FZD but translocates to the destruction complexes upon ligand binding. In the destruction complex Dsh's axin-binding ability might then directly or indirectly be employed to down regulate activity of the complex. Down modulation of GSK3β activity, for instance, has been proposed based on the finding that Dsh can also interact with the GSK3beta binding protein Frat (42, 43). Recent genetic evidence, however, seems to rule out this possibility since mice lacking all three Frat genes develop entirely normally (44). It is clear that the sequence of events that is initiated by receptor triggering and culminating in quenching the activity of the destruction complex awaits more detailed investigation.

When beta-catenin arrives in the nucleus, it binds to the N-terminus of members of the TCF/Lef family of HMG box containing-transcription factors (TCF1, Lef-1, TCF3 or TCF4) (45, 47). These TCF – or Lef/beta-catenin complexes form active transcription units because of the potent transactivation domain found at the C-terminus of beta-catenin (46). Thus, by binding to their recognition elements in promoters and enhancers of Wnt-responsive genes these DNA-binding proteins specify the sites in the genome where beta-catenin exerts its co-transcriptional activity (47). In the absence of Wnt signaling TCF/Lef proteins repress target genes through association with co-repressors like Groucho (48) rendering the chromatin structure inaccessible for the basal transcriptional machinery via recruited histone deacetylases (49). In case of active signaling this repression is relieved by replacing Groucho proteins for accessory molecules serving as positive effectors of Wnt target gene transcription (50). Two of these factors are the histone acetylase CBP/p300 (51) and the SWI/SNF component BRG1 (52). Legless (BCL9) and Pygopus could mediate further interactions between the beta-catenin-TCF complex and chromatin. Drosophila Legless or the two mammalian homologs BCL9.1 and BCL9.2 have binding domains for Armadillo/beta-catenin as well as for Pygopus. Legless/BCL9 may thus function as an adaptor between TCF/beta-catenin and Pygopus. Originally it was proposed that both Legless/BCL9 and Pygopus would function in the

nucleus as co-factors to enhance beta-catenin mediated transactivation (53, 54). Recently, a different mechanism was proposed to explain how Armadillo/beta-catenin is controlled by Legless/BCL9 and Pygopus. In this analysis (55), it was shown that the constitutive nuclear protein Pygopus functions as a nuclear anchor for Legless/BCL9. The presence of Legless/BCL9 in the nucleus would then serve to target beta-catenin to the nucleus. This targeting would reflect either Pygopus/Legless-BCL9 mediated nuclear import or nuclear retention. In addition to this issue of how exactly these proteins function in beta-catenin/TCF signaling future experiments will also have to determine whether these proteins are absolutely required for canonical Wnt signaling during development, self renewal of epithelia in the intestine, mammary gland and the hair follicle, as well as in colorectal cancer. Another scenario that warrants further exploration is the possibility that only a subset of Wnt target genes require these co-factors for their transcriptional activation. It seems reasonable to assume this because the potent transcriptional transactivation domain found at the C-terminus of beta-catenin can act independently of BCL9/Pygopus (56).

4.3 Wnt signaling provided by the niche

There are now several lines of *in vivo* evidence showing that proliferation of intestinal crypt cells is dependent on Wnt signaling activity. As mentioned before, removal of the HMG box transcriptional factor TCF4 depletes the epithelial layer of its stem cell compartment (18, 57). Blocking Wnt signaling at the level of Wnt interaction with the FZD/LRP complex in adult mice, as a different approach to investigate the role of this pathway on intestinal homeostasis, was presented by two groups. Pinto *et al* (58) genetically introduced the soluble Wnt inhibitor Dickkopf-1 (DKK)(59) into mouse intestinal epithelial cells. A similar type of experiment exploited adenoviral introduction of this inhibitor into the small intestine and colon of adult mice (60). Both experiments showed a severe reduction in growth of epithelial cells in both the fetal and adult intestine. These results were confirmed by depleting cells for beta-catenin by using, P450 regulated, Cre-mediated control of gene expression (60). *In vitro* studies, using colorectal cell lines in which downstream stages of the Wnt pathway were blocked by inducibly over expressing TCF protein lacking a binding domain for beta-catenin (termed dominant negative TCF), or siRNA for beta-catenin, again supported the assumption that in the absence of Wnt signaling intestinal epithelial cells arrest in the cell cycle (57, 62).

Whereas blocking the Wnt pathway results in cell cycle arrest, active cycling of intestinal epithelial cells is expected to be associated with accumulation of free cytoplasmic beta-catenin and its subsequent nuclear uptake. Immunohistochemical staining for beta-catenin shows that the protein is indeed not only present in the basolateral membranes of the epithelial cells but also in the nuclei of the proliferative cells in the crypt (57). Whether nuclear localization of beta-catenin is a hallmark of all proliferating cells in the crypt, including the stem cells, or only of TA cells remains unclear. The availability of an independent stem cell marker is essential in this respect. Musashi-1, a neural RNA-binding protein, has been proposed as such (63). It was isolated as a mammalian homologue of a *Drosophila* protein required for asymmetric division of sensory neural precursors and has been implicated in the maintenance of neural stem cells (64). Immunohistochemical staining for Musashi-1 in adult mouse small intestine confirmed the position of stem cells at position 4 – 5 just above the Paneth cells as classically detected by Potten using [^{3}H]-thymidine (65) but showed that also the columnar cells intermingled between Paneth cells express this protein.

From our present knowledge of the Wnt signaling cascade and the strong association identified between pathway inhibition and the absence of proliferation in the intestine, one would conversely predict that any mutation in components of this pathway resulting in the constitutive accumulation of beta-catenin leads to hyper proliferation of these epithelial cells. The most widely used animal model demonstrating this concerns the *Min* (Multiple intestinal neoplasia) mouse (66). *Min* mice, heterozygous for a nonsense mutation in the APC gene, stably express a truncated form of the APC protein from the affected gene. The truncated protein is unable to function properly within the destruction complex. On aging, these mice develop numerous adenomas, predominantly in the small intestine. Cells in the adenomas invariably show LOH (loss of heterozygosity) for the wild type APC allele (67) emphasizing the need for complete absence of full-length APC to allow accumulation of beta-catenin. A similar bi-allelic inactivation of the APC gene appears also to be required for the initiation of adenomas as seen in humans; these adenomas subsequently develop into colorectal cancers (CRC) (68). Recently a study was presented in which gene ablation of the negative regulator APC was analyzed in adult mice by using an inducible villin/Cre-mediated system (69). Despite the absence of APC in the villus epithelial cells, these differentiated cells did not re-enter the cell cycle. This distinct biological response of the proliferative compared to the differentiated cells also seems to apply to Paneth cells. Terminally differentiated cells seem to employ mechanisms to switch-off the mitotic instructions provided by beta-catenin/TCF signaling (see section on Wnt signaling and Paneth cell differentiation).

4.4. Genetic program driven by Wnt cascade effectors beta-catenin/TCF

4.4.1. Proliferation vs. differentiation

Our laboratory performed micro-array experiments to identify the TCF/beta-catenin target gene program, activated by Wnt pathway mutations, in CRC cells (57) (previous identified target genes reviewed in www.stanford.edu/~rnusse/wntwindow.html). Disruption of the pathway, by inducible expression of TCF proteins lacking beta-catenin binding function, revealed three major consequences. First, abrogation of Wnt pathway activity by the induction of dominant-negative TCF (dnTCF) resulted in a rapid entry into G1 arrest of the CRC cells. Secondly, the same genes switched off in this system are physiologically active in early intestinal polyps from *Min* mice, aberrant crypt foci from FAP patients and, significantly, also in the proliferative crypt compartment of

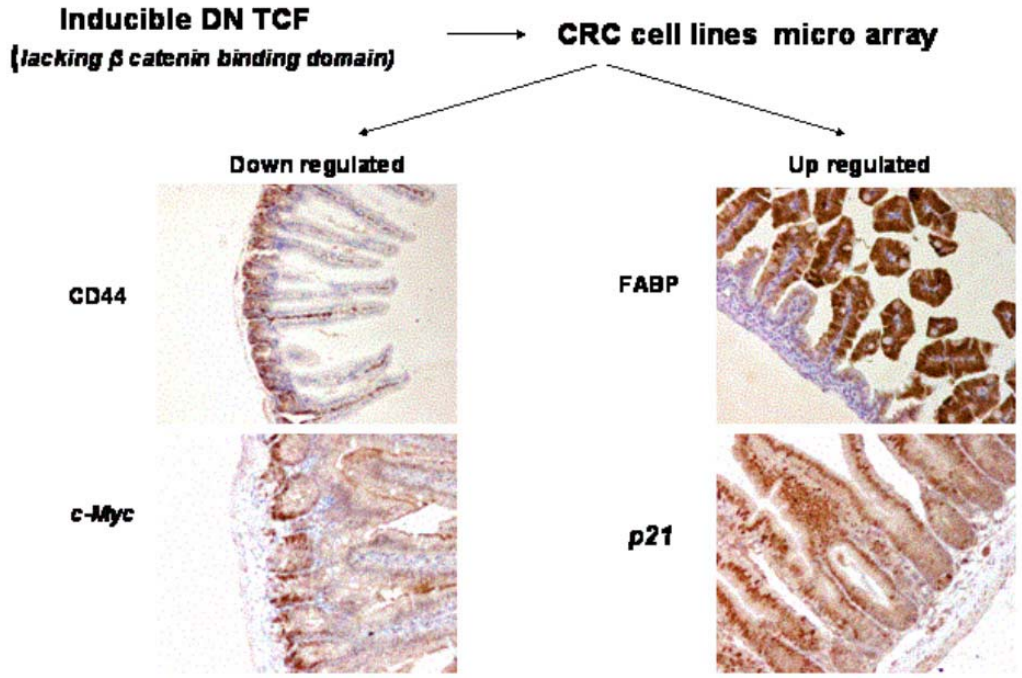

Figure 3. Interfering with Wnt signaling in CRC cell lines. The constitutively active Wnt pathway in CRC cells was inducibly blocked by introducing dominant negative versions of TCF (see ref. 57) and the effects on global gene expression evaluated using microarrays. This analysis, and subsequent validation of targets using immunohistochemical staining, reveals that genes switched off in this system are physiologically active in early intestinal polyps from Min mice, aberrant crypt foci from FAP patients and, significantly, also in the proliferative crypt compartment of the normal intestine. The expression patterns of two examples, CD44 and C-myc, are presented. The set of genes up regulated following loss of Wnt signaling appeared to be specifically expressed in the differentiated cells covering the villi in the normal intestine. The staining patterns for two examples, p21 and FABP, are shown.

the normal intestine. The third finding of this experiment was the detection of a set of indirect target genes whose transcription was found to be up regulated in response to silencing the pathway. These gene products appeared to be specifically expressed in the differentiated cells covering the villi in the normal intestine. These findings again designate the Wnt pathway as the driving force controlling proliferation of intestinal epithelial cells but also imply a role in the differentiation of this tissue (Figure 3).

The results obtained from our microarray studies identify the *c-MYC* gene product as a regulator of proliferation in these CRC cells. One observation supporting this is that the cell cycle arrest seen in dnTCF-silenced CRC cells can be overcome by introducing a source of Wnt-independent *c-MYC* in these cells. This protein has been implicated in the proliferative capacity of many cancers (70). Its cell cycle effector role in CRC cells has previously been identified (71) by introducing wild type Apc into CRC cells having truncating mutations in both Apc alleles. The down regulatory effect on the levels of be concomitantly reduced the levels of c-MYC. That the CRC cells represent crypt progenitors is reflected in c-MYC expression in the proliferating crypt cells of the normal intestine (57, 69). Moreover, introduction of CDK inhibitor p21cip1/waf1, a downstream effector of *c-MYC,* into cycling LS174 CRC cells blocks their proliferation (57). This is perfectly in line with the reported expression of $p21^{cip1/waf1}$ exclusively in the non-proliferating differentiated cells in epithelium of the colon (72) and small intestine (58). We concluded that *c-MYC* permits proliferation in CRC cells by repressing the synthesis of the CDK inhibitor $p21^{cip1/waf1}$. Silencing of the Wnt pathway in crypt progenitor cells de-represses transcription of the p21 cell cycle inhibitor leading to a stop in cell growth and induction of differentiation. Terminal differentiation in the intestine is intimately coupled to cell cycle arrest. It remains unclear, however, how both processes are linked. The cell cycle inhibitor $p21^{cip1/waf1}$ but also $p27^{kip1}$, another member of this family, have been closely linked to terminal differentiation in many systems (73). Although enforced expression of these inhibitors triggers intestinal differentiation *in vitro* (74, 75) there is no obvious phenotype in the intestines of mice with targeted $p21^{cip1/waf1}$ or $p27^{kip1}$ deletion (76, 77). Since co-operation occurs between different members of this family (78), there is a clear need to analyze animals deficient for multiple members of this family.

4.4.2. Wnt signaling and Paneth cell differentiation

Recent observations have led to the conclusion that cell differentiation in the intestinal epithelial layer,

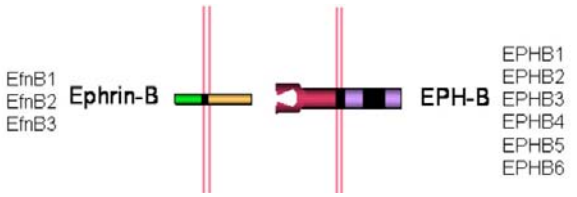

Figure 4. Overview of families of Eph-B – and Ephrin-B tyrosine kinase receptors. Direct cell-to-cell contacts, involving interactions between tyrosine kinase receptors of the Eph – and Ephrin families generate bi-directional signaling resulting in repulsion or homotypic adhesion between encountering cells (see ref. 84). These regulators of migration are grouped into subclasses A and B. EphA receptors bind A-type Ephrin ligands, while EphB receptor subfamily members bind to B-type Ephrin ligands.

although arising in the context of cell cycle arrest, does not necessarily occur in the absence of Wnt pathway activity. Paneth cells appear to move towards the bottom of the crypt where they meet the source of Wnt signals, whereas the other three types of differentiating cells move away from this source onto the villus. It has become clear that Paneth cells indeed sense the Wnt protein that is present in the crypt niche as they accumulate high levels of beta-catenin (69, 79). Wnt signal processing by the Paneth cells, however, does not drive proliferation but instead supports maturation of these cells (80). The first indication for Wnt-driven maturation of Paneth cells came from micro-array experiments comparing intestinal gene expression at E15.5 – E18.5 between TCF4 +/- and TCF-/- embryos performed in our laboratory (80). Obstruction of Wnt signal transduction led to loss of expression of a number of previously identified Tcf4 target genes (for example, Myb, Myc, Gpx2 and Cdx1). To our surprise this set of gene products also included a large panel of Paneth cell markers, despite the fact that these cells are morphologically absent during embryonic development and physically only appear two weeks after birth. Among these markers were several cryptdins (the mouse cryptdin family encodes nineteen of these antimicrobial proteins of which 1 – 6 are specific to Paneth cells (81)), MMP-7, peptidoglycan recognition proteins and Mpgc60. Cryptdin proteins and the metalloproteinase MMP-7 are functionally linked since Cryptdins are only active after processing by MMP-7 (81). The finding that this program is also active in the adenomas of APCmin mice further supports direct regulation of the MMP-7/Cryptdin program by the Wnt pathway. In addition, most human colorectal cancers express defensin-5 and 6, the functional counterparts of the cryptdins. Transient reporter assays based on the human defensin-5 promoter and chromatin immunoprecipitation studies unambiguously identified these genes as direct targets of TCF/beta-catenin controlled transcription. Wnt-induced induction of Paneth cell maturation, expression of cryptdins in adenomas and the direct transcriptional control by the Wnt pathway were also recently observed in mice upon loss of APC (69).

The remaining issue now was how Paneth cells and progenitor cells respond so differently to the same Wnt signal? Part of the answer was found during an '*in situ*' analysis for the presence of Wnt components during embryonic and postnatal life. The Wnt receptor FZD6 was found in all epithelial cells with the exception of Paneth cells, while FZD5 was specifically detected in crypt cells of embryonic and adult mice. Inducible gene deletion of LoxP-flanked FZD5 introduced into the intestine-specific K19Cre knock-in mice resulted in a mosaic pattern of the Cre enzyme throughout the intestinal epithelium from early embryonic stages into adult life. The morphologically distinguishable Paneth cells in adult animals, lacking FZD expression, displayed complete absence of nuclear beta-catenin and of cryptdin-1 mRNA (77) but positively stained for lysozyme, a Wnt-independent marker. These experiments justify the conclusion that Paneth cells reach full maturity through Wnt-induced expression of a program that includes MMP-7 and cryptdins. The FZD5 receptor mediates this effect but its role warrants more detailed analysis. One explanation for the difference in response of progenitor cells and presumptive Paneth cells to the same Wnt environment is that gene program specification is determined by the FZD5 receptor. Such a specificity, acquired during the initial step in transduction of the Wnt pathway is, however, difficult to reconcile with the observation that the MMP-7/Cryptdin associated program can apparently also be induced by downstream activation of the pathway as occurs in adenomas or colorectal cancers. Such a role for FZD5 seems further excluded by the notion that also the undifferentiated proliferating progenitor cells express this receptor, in addition to FZD6. Most likely, as is commonly seen in signaling during development, the state of the immature Paneth cell rather than the signal itself determines the type of response. The challenge now is to determine how the immature Paneth cell transduces its specific responsiveness.

4.4.3 Wnt signaling and cell migration

It has recently become evident that Wnt signaling is not only actively involved in the maturation of cells; its activity was also found indispensable for proper allocation of cells within the epithelial layer. For a better understanding of this issue we will first introduce the Eph and Ephrin families of receptor tyrosine kinases (82). Both receptor families have been found in all animal species. Although invertebrates have a very limited number of family members, there has been a major expansion of these families in vertebrates. Collectively, the family members appear to be expressed in all tissues during development. They have emerged as key players in the regulation of migration of cells during assembly and maintenance of organized tissue patterns. Based on their ligand binding specificity, these receptors are grouped into two subclasses. EphA receptors bind A-type Ephrin ligands, while EphB receptor subfamily members bind to B-type Ephrin ligands (83) (Figure 4). Direct cell-to-cell contacts, involving the appropriate receptor/ligand combinations, generate bi-directional signaling resulting in repulsion or homotypic adhesion between encountering cells. See Noren and Pasquale for a review discussing the biochemical pathways regulated by Eph receptors and Ephrins (84). Interactions and activation of Eph receptors and Ephrins can occur at the interface of complementary expression domains or within regions of co-expression or overlapping gradients.

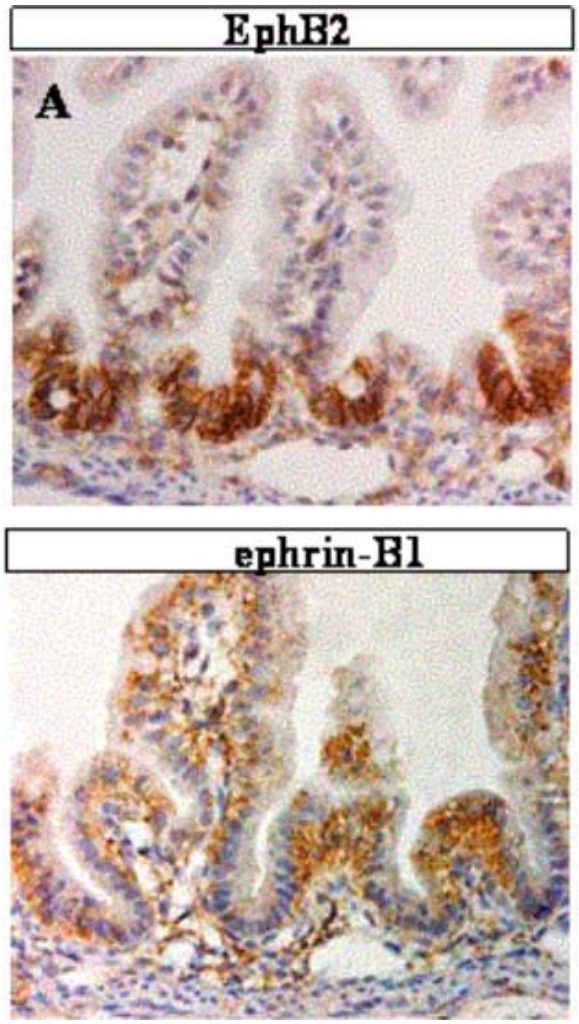

Figure 5. Expression of EphB2 and Ephrin-B1 in the intestine of neonatal mice. Immunohistochemical staining of intestinal sections reveals expression of EphB2 in intervillus pockets while the Ephrin-B1 ligand is expressed in all epithelial cells covering the villus. This complementary expression drives the formation of distinct domains comprising proliferating cells in the intervillus pockets and differentiated cells in the villus (see ref. 79).

The microarray experiment mentioned previously (57), analyzing gene expression in colorectal cells in the absence and presence of beta-catenin/TCF signaling, provided the first clue that Eph/Ephrin signaling is also operative in the homeostasis of self renewing tissues like the intestinal epithelium. EphB2 and EphB3 receptors were found among the genes regulated by dnTCF, which are physiologically expressed in the crypt region. In contrast, one of their putative ligands, Ephrin-B1, was among the genes expressed in the differentiated villus epithelial cells. Functional analysis of their role (79) started by monitoring their *in vivo* expression patterns. This showed, in accordance with the micro-array data, that both Eph receptors are mainly confined to the rapidly proliferating cells of the intervillus pockets in wild typeE18 embryos. Their expression was undetectable in TCF4-deficient mice, consistent with the regulatory transcriptional role of the Wnt pathway.

In the small intestine of newborn mice, EphB2 and EphB3 receptors were found in the intervillus pockets, while the Ephrin-B1 ligand was expressed by all cells in the villi (Figure 5). Cells near the crypt-villus boundary co-expressed receptors and ligand. This Eph/Ephrin complementary expression pattern proved important for the allocation of functionally distinct cells since in mice lacking both EphB receptors, proliferating ($Ki67^{+}$) and differentiating (Fatty Acid Binding Protein (FABP)) cells freely intermingled (79). This situation found in the newborn mouse, where receptor and ligand are expressed in complementary domains, is reminiscent of the establishment of segment boundaries in the developing brain where bidirectional signaling occurs between Eph and Ephrin expressing populations at the edge of odd and even rhombomeres (85)

In the intestine of adult mice a more complex pattern of Eph/Ephrin expression pattern was found (Figure 6). Both receptors and ligand, were now found in an overlapping complementary gradient. Eph-B2 was detected on all cells in the crypt, with the exception of Paneth cells, while its expression gradually decreased toward the top of the crypt. The EphB ligands Ephrin-B1 and B2 were found at highest level in the villi, while their expression diminished toward the bottom of the crypt. Thus, proliferative cells in the crypt co-expressed Eph-B2 receptors and their ligands in an inverse, position-dependent pattern. Two types of experiments suggested that this expression pattern determines the relative position of the cells within the crypt. First, in EphB2/-B3 double-mutant mice, sorting of Ephrin-B1 positive cells is severely disturbed. Ephrin-$B1^{high}$ and Ephrin-$B1^{low}$ cells were randomly positioned along the crypt. In a second approach, a villin promoter-driven dominant negative acting Eph-B2 (lacking the intracellular tyrosine kinase domain) was genetically introduced. This interference with Eph function resulted in a similar random positioning of Ephrin-B1 expressing precursor cells. A model emerges in which differentiating cells progressively down regulate Eph-B2 while at the same time up regulating Ephrin-B1 expression. The decrease in Eph-B2 expression favors migration up the gradient of Ephrin-B1 expressing cells. In contrast, a higher level of Ephrin-B1 drives cells down the Eph-B2 gradient. This ensures a unidirectional flow of migration. The overlapping gradients for expression of Eph and Ephrin, as seen here in the proliferative zone of the adult crypt, is reminiscent of the way topographic maps of neuronal connections are set up (reviewed in 86).

Paneth cells, as previously mentioned, migrate towards the putative Wnt source at the bottom of the crypt. Several experimental observations show that the surprisingly high level of Eph-B3 expression seen in these cells directly correlates with this specific migratory behavior. In Eph-B3 mutant mice, Paneth cells no longer move to the base of the crypt. Interfering with Wnt signaling in these cells by gene ablation of the FZD5

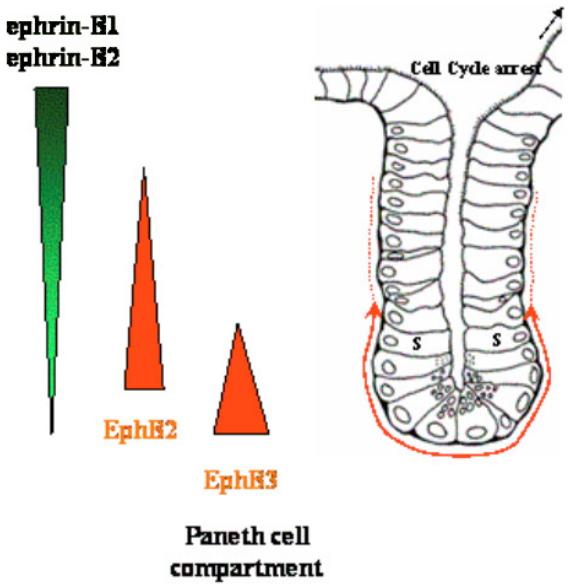

Figure 6. Schematic representation of reciprocal gradients of members of the EphB and EphrinB families in the adult intestine. A complex pattern of Eph/Ephrin expression pattern is found. In the intestine of adult mice. The overlapping gradients of EphB2/3 receptors and EphrinB1, 2 ligands determines the relative position of the cells within the crypt. A model is proposed (ref. 79) in which differentiating cells progressively down regulate Eph-B2 while at the same time up regulating Ephrin-B1 expression. The decrease in Eph-B2 expression favors migration up the gradient of Ephrin-B1 expressing cells. In contrast, a higher level of Ephrin-B1 drives cells down the Eph-B2 gradient. This ensures a unidirectional flow of migration of differentiating cells from the crypt onto the villus.

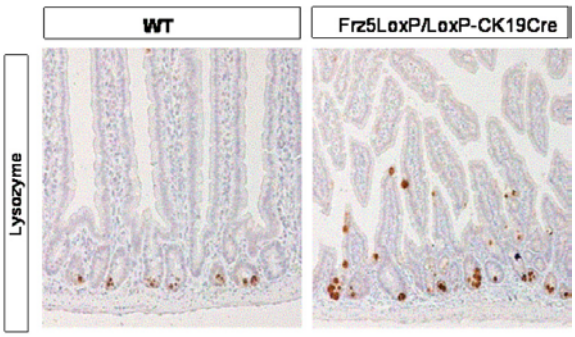

Figure 7. Straying Paneth cells in the intestinal epithelium of Frizzled5$^{-/-}$ mice. The high level of Eph-B3 expression seen in Paneth cells directly correlates with their downward migratory behavior. Interfering with Wnt signaling by gene ablation of the FZD5 receptor, that is specifically expressed by these cells, leads to a loss of Eph-B3 expression (80).The Paneth cells in these mice, identified by staining for Lysozyme, misposition along the crypt-villus axis. A similar random migratory behavior is also seen in Eph-B2^{neg}3^{neg} animals (79), as well as in Dickkopf expressing mice (58).

receptor (75), leads to a loss of the MMP-7/cryptdin program (which includes Eph-B3) and results in mis-positioning along the crypt villus axis (Figure 7). In fact, the migratory behavior of FZD5neg Paneth cells is indistinguishable from Eph-B3^{neg} cells. Random migratory behavior of Paneth cells is also seen in the villin promoter-driven transgenic DKK mice (53). This may provide the most direct evidence for Wnt signaling as the driving force for Paneth cell sorting. The high level of Eph-B3 expression in Paneth cells favors movement down the Ephrin-B1 gradient explaining their migration to the bottom of the crypt.

In conclusion, the bidirectional signaling between Eph/Ephrin that regulates repulsion and adhesion between cells, operates during development to set up sharp interfaces between two tissues or to control cell movements in a gradient. In the intestinal epithelium both types of organization are found. In the newborn mouse Eph/Ephrin interactions are instructive in setting up domains of proliferating and differentiating cells. In the adult mouse these receptor/ligand interactions are used to control graded movements of proliferating and differentiating cells. The low level of Ephrin-B1 expression seen in proliferating cells and the gradual increase in cells differentiating into enterocyte, goblet cell or enteroendocrine cell, drives the movement of these cells toward the villus but the same gradient drives migration of Eph-B3^{+} Paneth cells, the fourth differentiated cell type, in the opposite direction.

4.5 What are sources of Wnts?

Numerous observations imply the presence of a Wnt source involved in driving proliferation of crypt progenitor cells. Of particular relevance is the phenotype of mice in which the Wnt inhibitor DKK is expressed in the extra cellular space (58, 60) of Wnt-responsive cells in the intestine (87). Furthermore, the mispositioned Paneth cells in Eph-B3 mutant animals (79) invariably lack nuclear beta-catenin. This strongly indicates that nuclear accumulation of beta-catenin is a cell non-autonomous process, which depends on the position that the cells adopt along the crypt-villus axis. Indeed, several Wnts are expressed along the intestinal tract during mouse and chicken development (88, 89). In a recent report (90) the expression pattern of all Wnts, FZDs, low-density lipoprotein receptor-related proteins, Wnt antagonists, and TCF factors in the murine small intestine and colon as well as adenomas were investigated. This study predicts a much broader role for Wnt signaling in gut development and homeostasis than was previously anticipated from available genetic studies.

5. COUNTERACTING WNT SIGNALING

Ample evidence has accumulated identifying the Wnt pathway as the driving force for the proliferation of progenitors in the crypt compartment. In the same compartment, this pathway was now also found responsible as driving force for maturation of Paneth cells and for directing cells to their functional locations. We have seen that Eph-B3^{neg} Paneth cells, displaying high levels of nuclear beta-catenin whilst occupying their normal crypt

position, lose this characteristic when they cross the crypt-villus border. Silencing of the Wnt cascade at this border could simply be the consequence of the dissipation of the Wnt signal, but might also be actively induced by signals from other pathways. Several experimental observations associate the 'transforming growth factor-beta' (TGF-beta) pathway with growth inhibition in the intestine and may thus represent an example of a Wnt-counteracting pathway. This superfamily of soluble growth regulatory factors has been found to be involved in various biological processes including embryonic development, angiogenesis, proliferation and differentiation (91). This large family of cytokines comprises TGFbeta members and bone morphogenetic proteins (BMPs). A common mechanism of signaling applies to all superfamily members. Ligand binding brings a type I and type II serine-threonine kinase receptor together, resulting in the phosphorylation of the type I receptor by the type II receptor. Intracellular R-SMAD proteins (SMAD 1,2,3,5 and 8) become phosphorylated by activated type I receptors and associate with the co-SMAD SMAD4, and translocate to the nucleus. In the nucleus the R/co-SMAD complex interacts with co-activators or co-repressors to regulate transcription of target genes.

Signaling components of the TGFbeta family members have been detected in the intestinal layer and appeared mainly localized to the differentiated epithelial cells (92) In intestinal cells in culture, TGFbeta triggers a strong cytostatic response (93). However, most CRC cell lines are refractory to the inhibiting effects of TGFbeta owing to inactivating mutations in the pathway (94, 95). Reintroduction of a wild type form of the mutated component recovers the cytostatic response (96). These observations imply a growth suppressing effect of this pathway in the intestine and would confer a growth advantage of mutant cells. Although several models have been generated to explore the role of the TGFbeta members of this superfamily in development, their *in vivo* role has not yet been clearly established (reviewed in 97).

The situation is different for the BMP superfamily, of which there are more than twenty members. Two recent studies imply a role for BMP signaling in counterbalancing the Wnt pathway specifically in intestinal stem cells. BMP proteins specifically signal through R-SMAD1,5 and R-SMAD8. BMP-4 is expressed in the intervillus mesenchyme of adult mice and phosphorylated SMAD1, 5 and SMAD8 are found in the villus epithelium, indicative of paracrine signaling from the mesenchyme to the intestinal epithelium (98). In the first report (98), transgenic mice are described expressing Noggin, an antagonist of BMP signaling, in the intestinal epithelium. These animals develop a syndrome that is indistinguishable from Juvenile Polyposis Syndrome, an autosomal dominant hereditary polyposis syndrome, with increased risk of gastrointestinal malignancy. This syndrome is heterogeneous, though in most cases mutations are found in the BMP receptor BMP1RA, or the co-SMAD, SMAD4 (99). The inhibition of the BMP pathway results in ectopic crypt development, perpendicular to the crypt-villus axis. In the second report (100), analyzing conditionally-inactivated BMPR1A mice, evidence was found that BMP signaling may have a role in preventing stem cell renewal by inhibition of beta-catenin accumulation. Mutant mice developed characteristic polyps containing increased numbers of crypts and a 5-fold increase in the number of progenitor cells in comparison to wild type mice. The inhibitory effect on beta-catenin appears to be mediated via the dual protein and lipid phosphatase, PTEN. Mutations in this tumor suppressor gene (101) cause Cowden disease (102), another syndrome characterized by polyposis, and are also found in gastrointestinal cancers (103) This enzyme is known to down regulate the activity of phosphatidylinositol-3 kinase PI3K), which, through Akt (a serine-threonine kinase), promotes cell-cycle progression. Thus, PTEN inhibits Akt activity and the signals downstream of Akt. Conversely, inactivation of PTEN has been reported to activate Akt resulting in nuclear localization of beta-catenin (104). Detection of the inactivated (phosphorylated) form of PTEN, P-PTEN, and of phosphorylated Akt (P-Akt) specifically in BrdU retaining intestinal stem cells further supported this model. The presence of both enzymes in stem cells correlated with the presence of nuclear beta-catenin and with the activity of a TCF/beta-catenin-driven GFP reporter. Treatment of *ex vivo* cultured intestines with Noggin confirmed the regulatory role of P-PTEN and P-Akt on the sub cellular localization of beta-catenin. Endogenous Noggin was found to be expressed in the submucosal region adjacent to the crypt bottom and in a few cells in or around the stem cell position. These studies suggest that BMP signaling may have a role in inhibiting stem cell self renewal. Self renewal would be promoted by beta-catenin but inhibited by the BMP4/PTEN/Akt pathway. A similar role for PTEN was found in the regulation of embryonic and neural stem cells (105, 106). Abrogation of the BMP signal, through the action of Noggin, would generate fully active beta-catenin leading to stem cell division. The reported requirement of Noggin-Wnt coordination for initiation of the hair cycle (107) further supports this idea.

Another class of signaling molecule, which may oppose the effects of Wnt signaling in the intestine are the Hedgehogs (Hh). Secreted Hh proteins act through three different transmembrane proteins on Hh-responsive cells: Smoothened (SMO), Patched (PTCH) and Hh interacting protein (HIP). In the absence of ligands, PTCH is believed to block the activity of SMO. Upon binding of any of the Hh family members to the PTCH receptor, SMO inhibition is released. This event triggers a signaling cascade that culminates in the translocation of members of the GLI family of Zn-finger transcription factors from the cytoplasm to the nucleus. Three members of this family are encoded in the human genome: GLI-1, GLI-2 and GLI-3. Upon nuclear translocation, they drive the expression of target genes in Hedgehog-responsive cells (108). Hedgehog ligands act as morphogens during development as they control cell fate specification in a concentration-dependent manner. Sonic-hedgehog (Shh) and Indian-hedgehog (Ihh) are expressed in the epithelial layer and control the concentric tissue architecture of the gut (109, 110). Hedgehog pathway mutations are associated with multiple malformations along the gastrointestinal tract (110, 111,

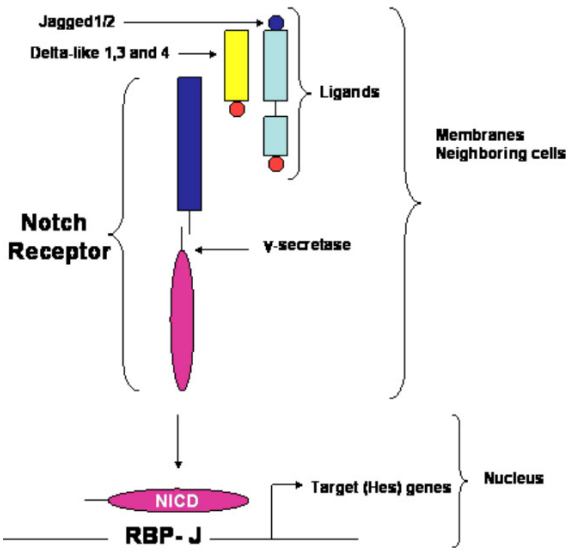

Figure 8. Simplified representation of Notch signaling. Binding of membrane bound Jagged1/2 or Delta-like 1,3 or 4 ligands with Notch receptor(s) ultimately leads to the action of a gamma-secretase. The Notch IntraCellular Domain (NICD) that is released by this enzymatic step enters the nucleus. Binding to the RBP- J transcription factor ultimately leads to activation of Hairy Enhancer of Split (HES) genes (Review, see ref. 116).

and 112). There is only limited information on the role of Hh signaling in the homeostasis of the adult intestine. A recent report describes Ihh and PTCH expression in rat colon. Cyclopamine, a drug that prevents binding of Hh proteins to SMO inhibited cell differentiation. In contrast, incubation of HT29 colorectal cells with recombinant Shh peptide induced differentiation of these cells. This treatment, moreover, was found to abrogate TCF/beta-catenin signaling in these cells. In line with a differentiation promoting and Wnt inhibiting role of Hh signaling, dysplastic cells in adenomas from FAP patients show lost Ihh expression. DnTCF induction in the colorectal cell line DLD1 induced Ihh expression supporting a Wnt inhibitory effect on Ihh expression. The model proposed in this study suggests that in the Wnt-responsive crypt progenitors Ihh expression is suppressed. Cells not receiving Wnt signals would upregulate Ihh that in turn may induce differentiation of these cells (113).

6. NOTCH AND WNT PATHWAYS CO-ACTING IN INTESTINAL PROGENITOR CELLS

Inhibition of the Wnt pathway either by deletion of TCF4 (18), beta-catenin (61) or by the transgenic or viral expression of the soluble Wnt inhibitor DKK (58, 60) blocks the proliferative state of intestinal progenitors. Recently, evidence was obtained that Notch signaling is also required to maintain proliferative undifferentiated crypt cells. However, Notch signaling appears to ascertain the proliferative status of crypt cells by suppressing differentiation towards a Goblet cell fate.

Notch signaling is an evolutionarily conserved mechanism that transmits signals between cells that are in direct contact with one another. In eukaryotes, this pathway is vital for a seemingly endless variety of developmental cell-fate choices (114). The last few years have witnessed an explosion of new functions for this pathway also during adulthood. In its simplest form (Figure 8), signaling is triggered when the Notch receptor binds to ligands of the Delta or Serrate (known as Jagged in vertebrates) families. Both Notch and its ligands are cell-surface type-I transmenbrane proteins. Upon ligand binding, Notch receptors undergo successive proteolytic cleavages that ultimately lead to the release of the Notch Intra Cellular Domain (NICD), the active form of the receptor. The final cleavage is catalyzed by a γ-secretase acting within the Presenillin complex (115). NICD translocates into the nucleus and assembles into a ternary complex with the RBP-J (CSL or CBF1) DNA binding protein (116). Notch receptors 1-4 and its ligands Delta 1, 3,4 and Jagged 1,2 are all expressed in the mouse small intestine, although at different levels and with varying spatial and temporal distribution (117, 118). The best-characterized Notch target genes are the hairy/enhancer of split (HES) bHLH (119) and Achaete-Scute transcriptional repressors (120). Examples of these detected in intestinal epithelial cells are Hes1, 5, 6, 7, Math1 and Neurogenin (114, 121, and 122). The analysis of mice deficient for the basic helix-loop-helix (bHLH) proteins Hes-1 (123), Math1 (121) and Neurogenin-3 (122) have indirectly implicated the Notch pathway in the regulation of the earliest intestinal cell fate decisions. Toxicological studies on rodents with inhibitors for the γ-secretase that is responsible for release of the NICD, revealed increases in the size and number of mucus-secreting goblet cells, again giving indirect support for the control of intestinal cell fate by Notch (124). A study in zebrafish described increases in secretory cells at the cost of absorptive cells in the intestines of animals that are mutant for the Notch ligand Delta and *mindbomb* (125). Two recent mouse studies provide the most direct and convincing evidence that Notch controls differentiation of progenitor cells in the intestine. In the first report (126) the role of the Notch pathway was directly assessed by crossing floxed RBP-J mice with P450 –Cre and villin-Cre transgenic mice. Another approach involved blocking of Notch signaling by the γ-secretase inhibitor DBZ (124). Both approaches elicited essentially the same effects on intestinal epithelial cells. In uninduced/untreated intestines, Hes-1 is specifically expressed in the crypt progenitor cells while Math-1 is only detected in secretory cells. In the Notch-silenced intestines the expression of Hes-1 is completely lost resulting in derepression of Math-1 and its consequent expression throughout the epithelial layer. PAS staining revealed that these Math-1^+ cells all had converted to post mitotic Goblet cells. Immunohistochemical detection for the proliferation markers Ki-67 and BrdU confirmed that these differentiated cells had arrested in the cell cycle. In contrast, staining for nuclear beta-catenin showed that the Wnt pathway remained active in these cells. In the second report a gain of function approach was taken by producing double transgenic mice, carrying both villin-Cre and Rosa-Notch (127). The resulting constitutive Notch activity in all cells of the epithelial lining generated a

phenotype reciprocal to that reported for the loss of function study (126). All cells expressed Hes-1, leading to complete repression of Math-1. In accordance with the reported phenotype for Math-1 mutant mice (121), no goblet cells were detected. The number of proliferating cells had greatly increased in comparison to uninduced animals. Although this clearly points to an expansion of the progenitor pool the experiments, due to the lack of proper markers, do not discriminate between an increase in the number of stem cells or TA cells. The growth of cells in the villus region, outside the presumptive Wnt source, raises the issue of how this cell cycling is regulated. Assuming that these cells have no nuclear beta-catenin, Notch-directed proliferation (128) would provide the simplest explanation. Both studies emphasize that expression of Hes-5 (mainly by Paneth cells) does not respond to the modulation of Notch activity although this gene is a target for Notch signaling in the context of neuronal development (129). The influence seen on the proliferative status of progenitors, in animals in which Notch signaling was either completely blocked or instead constitutively active, was absent in Hes-1 mutant mice. The explanation for the more severe phenotype in the Notch-effected animals may be found in the involvement of Hes-6. Expression of this gene proved sensitive to Notch silencing in this tissue (126). Hes-6 may, in addition to Hes-1, also repress Math-1 but might also regulate other genes involved in secretory cell lineage differentiation. The analysis of epithelial cells in the NICD transgenic animals (127) implicates the Notch pathway in the differentiation of enteroendocrine cells and Paneth cells. Decrease of the number of enteroendocrine cells was based on staining for chromagranin or applying Grimelius silver stain. Decrease in the number of Paneth cells was evaluated by RT-PCR for cryptidin-1. Increases in the frequencies of these cell types, using lysozyme as marker for Paneth cells and synaptophysin for enteroendocrine cells, were detected in the RBP-J deficient animals. These reports imply that Notch signaling within the crypt, as deduced from Hes-1 expression in these cells, is essential to maintain the undifferentiated state of the crypt progenitors. The remarkable symmetry between crypt proliferative progenitor cells and colorectal cancer cells in terms of expressed Wnt target genes (57) extends also to the Notch pathway. Treatment of APC^{min} mice with DBZ leads to loss of Hes-1 and gain of Math-1 expression within the adenomas. Cells arrest in the cell cycle (Ki-67) and differentiate into goblet cells (PAS) while the Wnt cascade remains active (nuclear beta-catenin). Because treatment intensity and duration were limited in these experimental animals due to the concurrent changes in the normal epithelial layer, conversion into goblet cells within the adenomas was only partial. The fact, however, that an active Notch pathway within adenomal cells is essential to maintain the undifferentiated state provides a proof of principle for the notion that γ secretase inhibitors could be developed into therapeutic modalities for colorectal neoplasia.

The concerted action of the Wnt and Notch pathways in crypt proliferative progenitors and colorectal cancer cells raises the question of how these pathways are regulated. One intriguing explanation derives from the epistatic relationship between the Notch and the Wnt pathways postulated in vertebrate somitogenesis, in which Lef1 was found to act upstream of Delta/Notch signaling (130). Delta1-like was also found as a TCF/beta-catenin target in microarrays performed on colorectal cancer cell lines (57) and is expressed in mouse intestinal epithelial cells (117).The same micro-array did not, however, reveal a change in the expression level of Hes-1, the key effector of Notch activity. Analysis of intestines from TCF4-/- animals for expression of Hes-1 should reveal whether such a link exists in vivo.

7. GENETIC ALTERATIONS THAT LEAD TO INAPPROPRIATE REGULATION OF THE WNT PATHWAY INDUCE COLORECTAL CANCER

Many colon cancer syndromes have been characterized by their specific phenotypic, histological and genetic changes. The most common and well studied colon cancer syndromes are familial adenomatous polyposis (FAP, autosomal recessive) and hereditary nonpolyposis colorectal cancers (HNPCC, autosomal dominant), which are caused by mutations in the APC and mismatch repair (MMR) genes, respectively. Other colon cancer syndromes, all characterized by hamartomatous polyps and inherited in an autosomal dominant fashion, include Peutz-Jehgers syndrome (PJS), juvenile polyposis syndrome (JPS), hereditary mixed polyposis syndrome (MHAP) and Cowdens syndrome.

7.1 Initiation of Wnt-driven CRC

Approximately 15% of CRCs with involvement of APC occur in the context of a familial, i.e. inherited, predisposition (FAP) whereas the remainder (85%) arises sporadically. FAP individuals develop hundreds to thousands of adenomatous polyps of which a subset invariably progresses to malignant cancers (131). Controversy still exists over the origin of the cells from which these tumors arise. The ‘bottom-up’ model claims that the tumors arise from transformed stem cells at the bottom of the crypt. Expansion of the transformed stem cells towards the surface epithelium would occur during progression to adenoma (132). Alternatively, in the top-down model, the adenomas are thought to arise from transformed cells present within the surface epithelium. Expansion would occur by lateral migration and downward growth into the crypt (133).

Germline (loss-of-function) mutations in the APC gene were found to be the essential genetic event responsible for initiation of FAP. Subsequently, somatic mutations in the same gene were found to be associated with the majority of sporadic CRCs and benign intestinal neoplasms. In most of these cases the mutations yield a truncated APC protein (134). APC is considered a classic tumor suppressor gene, as both alleles must be inactivated for loss of tumor suppressing activity. For FAP and sporadic CRC patients, the molecular mechanisms underlying the lack of active APC protein can be a second truncating mutation or, more typically, loss of the second allele (LOH). The link between loss of APC function and CRC formation has been confirmed in experimental mouse

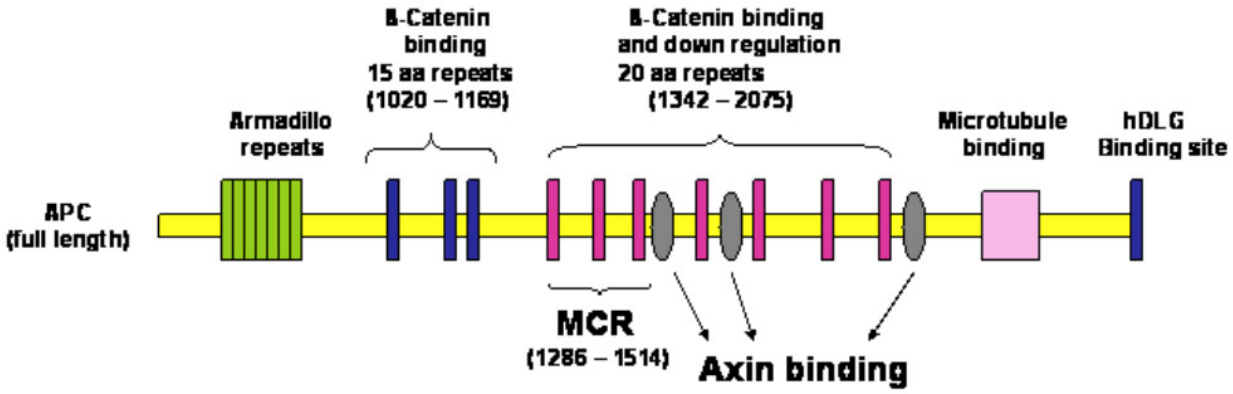

Figure 9. Structural features of the APC protein. Most of the mutations in APC occur in the Mutator Cluster Region (MCR) and create truncated proteins that lose the beta-catenin regulatory activity that is located in the 20 aa repeats. Alternatively, loss of control over the Wnt pathway may also be due to loss of Axin binding sites. Binding sites for a number of other important interaction partners are indicated.

models. The first and still most widely used model is the Min (multiple intestinal neoplasia) mouse (66). These mice express a truncated APC protein due to a nonsense mutation at codon 850. Homozygosity of this mutation leads to death of the embryos *in utero* at 8 days post coitus. Heterozygous mice phenotypically reflect the intestinal manifestations observed in FAP patients, i.e. development of numerous adenomatous polyps. In contrast to what is seen in FAP patients, the polyps predominantly occur in the small intestine rather than in the colon. The reason for this is as yet unknown. All of these adenomas harbor allelic loss of the wild-type APC allele whereby only the APC allele carrying the *Min* mutation remains in the intestinal adenomas (67). Similar findings were reported for mutations in APC leading to truncations at codons 1638, 716, 1309 and 474 respectively (135 – 138).

Wildtype APC appears as a large protein (315 kDa) containing several conserved regions for which at least 10 interaction partners have been reported. Established interaction partners (Figure 9) include beta-catenin (139), axin (140), microtubules (141), the microtubule binding protein EB1 (142), and the human homolog of the Drosophila *discs large* tumor suppressor protein (143). As a component of the beta-catenin destruction complex, the APC protein has a critical role in the turnover of cytoplasmic levels of beta-catenin, the key effector of the canonical Wnt signaling pathway. The accumulation of nuclear beta-catenin observed in CRC cells from FAP patient reveals that the remaining truncated APC protein is unable to function as negative regulator for the presence of cytosolic beta-catenin. The central region of APC contains multiple beta-catenin interaction motifs, including three 15 amino acid (15aa) repeats and seven 20 amino acid (20 aa) repeats. Three SAMP repeats, interspersed among the 20 aa repeats, mediate APC's interaction with Axin (Figure 9) (144). Although both the 15 aa repeats as well as the 20 aa repeats bind to the same region of beta-catenin (145), only the 20 aa repeats seem involved in its down regulation (146). The affinity of the 20 aa repeats for beta-catenin binding increases 300 – to 500-fold upon phosphorylation suggesting that this post translational modification functions as a critical switch for APC function. The spectrum of mutations in APC, as detected in FAP patients as well as in sporadic cases of CRC, clusters in the 20-amino acid-repeat region. This suggests that the selective pressure acts against the presence of beta-catenin regulatory domains, although selection against Axin binding can not be excluded. Mice lacking wild-type APC but expressing a truncated APC protein retaining a single Axin binding site are viable and do not develop neoplasia in the intestine (147).

Although both copies of the APC gene are typically inactivated in colorectal cancers, it remains possible that a mutant truncated APC may contribute to cancer progression. Transgenic expression of a mutant form of APC (delta 716) in a wildtype background does not result in cancer-prone mice and thus argues against dominant negative effects by truncated APC proteins (148). It may, however, be that truncated forms of APC in a background lacking wildtype APC contribute to tumor progression. This has been suggested by an analysis of somatic APC point mutations and LOH in colorectal adenomas from FAP patients (149). It was observed that when germline mutations resulted in truncated APC proteins without any of the seven 20 aa repeats the corresponding somatic mutations led to retention of at least one of these repeats. Conversely, when the germline mutation resulted in a truncated APC protein retaining one 20 aa repeat, most second hits had removed all 20 aa repeats. This non-random distribution of somatic hits has been interpreted as the result of selection for more advantageous mutations during tumor formation. Selection for APC genotypes that are likely to retain some activity in down regulating beta-catenin signaling may be optimal for tumor formation. There is a second option in which truncations in the APC protein may lead to loss of down modulation of Wnt signaling activity in CRC cells. This is related to its proposed ability to export beta-catenin from the nucleus thereby depleting TCF4 from its transcriptional co-activator (150, 151). Mutations in other components of the Wnt

pathway, i.e., in beta-catenin (152, 153) and conductin (154) can result in inappropriate signaling via TCF and initiation of CRC.

7.1.1 EphB receptors and colorectal cancer progression

We described the phenotypes in the small intestine of mice deficient for the beta-catenin/TCF target genes EphB2 and or EphB3 (see section on Wnt signaling and cell migration). This indicates that these receptor tyrosine kinases are involved in regulation of cell migration. The repulsive forces that underlie the migratory behavior of the intestinal epithelial cells result from binding of EphB molecules to membrane-bound ligands of the Ephrin family on neighboring cells. There are now strong indications that these mechanisms not only operate in healthy tissue but that these repulsive forces also limit progression beyond the early stages of CRC. The initial indications for a tumor suppressive role of EphB molecules were found in an analysis of expression of EphB2, EphB3 and EphB4 in a collection of human CRC samples at different stages of malignancy (155). Early lesions showed expression of these receptors at equivalent levels to that of normal crypt progenitor cells. Strikingly, the majority of colorectal carcinomas contained more than 50% EphB negative cells despite clear nuclear beta-catenin localization. Two independent reports (156, 157), analyzing the prognostic value of EphB expression levels in humans human cancer samples, reinforce the idea that EphB expression correlates inversely with patient survival. More direct evidence indicating that progression towards the carcinoma stage benefits from loss of EphB expression was found in APC^{min} mice genetically engineered to have low EphB activity (155). Accelerated tumorigenesis resulted in higher numbers and larger colorectal polyps. In addition, 20 % of these neoplasms were very large adenocarcinomas that rarely arose in control littermates.

The mechanism of silencing of EphB expression in CRC, which should act in a dominant fashion over beta-catenin/TCF signaling, remains uncharacterized. Some examples were found where genetic alterations lead to inactivation of individual EphB molecules. A small proportion of prostate cancers, for instance, contain point mutations in EphB2 (158) (159). LOH for EphB2 was seen in a significant portion of CRC patients (160) but no point mutations were detected in the remaining alleles. Promoter methylation as a silencing mechanism has yet to be evaluated. It seems, however, unlikely that the coordinated silencing of all three EphB genes during cancer progression, as was reported by Batlle *et al*. (155), can be accounted for by promoter methylation.

8 PERSPECTIVE

The intestinal epithelial layer is a tissue that is characterized by a rapid and perpetual self-renewal along the crypt-villus axis. Renewal requires division of multipotent stem cells, followed by transit amplification, and differentiation of daughter cells into specialized absorptive and secretory cells. This review summarizes the importance of the canonical Wnt pathway in diverse aspects of the homeostasis of this tissue. Although numerous core components of the pathway have been identified, the precise role of a number of them is still unclear. Examples include the Dsh protein and the mechanism by which it mediates inactivation of the beta-catenin destruction complex following binding of Wnts to their receptors. The exact requirement of Pygopus and BCL9 proteins in translating Wnt signals into active transcription provides a second example. The main role of canonical Wnt signals in the intestine is to drive proliferation of epithelial progenitor cells. Consequently, in the absence of Wnts, progenitor cells differentiate. Micro-array analysis has now produced extensive lists of genes regulated by beta-catenin/TCF signaling. It seems highly unlikely that these candidates are all primary targets for beta-catenin/TCF. The future challenge will be to unravel the presumed hierarchy within these lists. Chromatin immunoprecipitation experiments, using TCF - or beta-catenin-specific antibodies will likely be instrumental in addressing this issue. Apart from its role in prompting proliferation, recent findings also implicate Wnt activity in epithelial cell migration/sorting within the crypt-villus axis. Research in this area will undoubtedly focus on dissecting the molecular mechanisms by which EpH/Ephrin signaling controls cell migration within the intestine. An unexpected finding was the involvement of beta-catenin/TCF mediated transcription in the terminal differentiation of Paneth cells. This, moreover, takes place in the bottom of the crypt where the local Wnt source is at the same time implemented for the maintenance of undifferentiated progenitors. The question to be addressed now is how different cells, residing in the same Wnt containing environment, display such different phenotypic outcomes. In the context of CRC, Wnt signaling on one hand promotes proliferation/prevents migration (crypt initiation of cancer), while on the other hand; canonical Wnt signaling results in decreased proliferation/induced cell migration (invasive front of CRC)(see review from T. Brabletz in this issue and ref. 161). The challenge, in more general terms, is to decipher which cells are responsible for the production of Wnts in the intestine and which combinations of Wnts and FZD receptors mediate the various responses observed. Maintenance of progenitor cells requires active Wnt signals but also input from the Notch pathway. What exactly are the relative contributions of these signals and how is their action co-ordinated? Is Notch signaling only essential for the generation of TA cells or does it also support the long-term maintenance of the stem cell? To be able to answer these questions, we will first have to morphologically define the stem cells. Subsequent isolation of these cells would then simplify the manipulations needed to answer these questions. Artificial inactivation of Notch signaling in the intestinal layer by gene ablation, or by the application of pharmaceutical compounds, leads to a block in proliferation and a complete conversion of progenitors towards the Goblet cell lineage. These experiments revealed that in the absence of Notch signals undifferentiated epithelial cells differentiate towards the secretory lineages. The self-renewing capacity of the intestinal epithelium has been closely linked to the initiation of malignant transformation. We have reviewed how mutations in various components of this cascade initiate colorectal cancer. The most recent findings

highlight how the EpH/Ephrin system controls the adenoma-carcinoma transition. More knowledge should be obtained to address how these genes are silenced during progression of adenomas towards the carcinoma state. Both the observations that proliferating progenitors convert to post-mitotic Goblet cells as well as the involvement of EpH/Ephrin signaling in the progression of colon cancer may supply us with new therapeutic strategies to control this important cancer.

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10 REFERENCES

1. Kedinger M., I. Duluc, C. Fritsch, O. Lorentz, M. Plateroti & J. N. Freund: Intestinal epithelial-mesenchymal cell interactions. *Ann. N.Y. Acad. Sci.* 859, 1-17 (1998)
2. Roberts D. J.: Molecular mechanisms of development of the intestinal tract. *Dev Dyn.* 219, 109 – 120 (2000)
3. Wells J. & D. A. Melton: Vertebrate endoderm development: *Annu. Rev Cell Dev. Biol.* 15, 393 – 410 (1999)
4. Potten C. S. & M. Loeffler: Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 110, 1001-1020 (1990)
5. Cheng H.: Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. II. Mucous cells. *Am J Anat* 141, 481-502 (1974)
6. M. Hocker & B. Wiedenmann: Molecular mechanisms of enteroendocrine differentiation. *Ann. N.Y. Acad. Sci*. 859, 160-174
7. Porter E. M., C. L. Bevins, D. Ghosh & T. Ganz: The multifaceted Paneth cell. *Cell Mol. Life Sci.* 59, 56-170 (2002)
8. M. Garabedian, L. J. Roberts, M. S. McNevin & J. I. Gordon: Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice: *J. Biol. Chem.* 272, 23729-23740 (1997)
9. Potten C. S., W. J. Hume, P. Reid & J. Cairns: The segregation of DNA in epithelial stem cells. *Cell* 15, 899-906 (1978)
10. Potten C. S., G. Owen & D. Booth: Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J. Cell Sci.* 115, 2381-2388 (2002)
11. Merok J. R., J. A. Lansita, J. R. Tunstead & J. L. Sherley: Co-segregation of chromosomes containing immortal DNA strands in cells that cycle with asymmetric stem cell kinetics. *Cancer Res.* 62, 6791-6795 (2002)
12. Hermiston M. L., M. H. Wong & J. I. Gordon: Forced expression of E-Cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for non autonomous regulation of cell fate in a self-renewing system. *Genes Dev.* 10, 985-996 (1996)
13. Schmidt G. H., D. J. Winton & B. A. Ponder: Development of the pattern of cell renewal in the crypt-villus unit of chimaeric mouse small intestine. *Development* 103, 785-790 (1988)
14. Yatabe Y., S. Tavare & D. Shibata: Investigating stem cells in human colon by using methylation patterns. *Proc. Natl. Acad. Sci. USA* 98, 10839-10844 (2001)
15. Cheng H. & C. P. Leblond: Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types: *Am. J. Anat.* 141, 537-561 (1974)
16. B. A. Ponder, G. H. Schmidt, M. M. Wilkinson, M. J. Wood, M. Monk & A. Reid: Derivation of mouse intestinal crypts from single progenitor cells. *Nature* 313, 689-691 (1985)
17. Novelli M. R., J. A. Williamson, I. P. Tomlinson, G. Elia, S. Hodgson, I. Talbot, W. F. Bodmer & N. A.vWright: Polyclonal origin of colonic adenomas in an XO/XY patient with FAP. *Science* 272, 1187-1190 (1996)
18. Korinek V., N. Barker, P. Moerer, E. van Donselaar, G. Huls, P. J. Peters & H. Clevers: Depletion of epithelial stem-cell compartments in the small intestine of mice lacking TCF-4. *Nat. Genet.* 19, 379-383 (1998)
19. Rijsewijk F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel & R. Nusse: The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell* 50, 649-657 (1987)
20. Wodarz A. & R. Nusse: Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* 14, 59-88 (1998)
21. Willert K., J. D. Brown, E. Danenberg, A. W. Duncan, I. L. Weissman, T. Reya, J. R. Yates III & R. Nusse: Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448-452 (2003)
22. Kadowaki T., E. Wilder, J. Klingensmith, K. Zachary & N. Porimon: The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes & Dev*. 10, 3116-3128 (1996)
23. Huang H. C. & P. S. Klein: The Frizzled family: receptors for multiple signal transduction pathways. *Genome Biol.* 5, 234-239 (2004)
24. Pinson K. I., J. Brennan, S. Monkley, B. J. Avery & W. C. Skarnes: An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407, 535-538 (2000)
25. Wehrli M., S. T. Dougan, K. Caldwell, L. O'Keefe, S. Schwartz, D. Vaizel-Ohayon, E. Schejter, A. Tomlinson & S. Ninardo: Arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* 407, 527-530 (2000)
26. Muller H., R. Samanta & E. Wieschaus: Wingless signaling in the Drosophila embryo: zygotic requirements and the role of the genes. *Development* 126, 577-586 (1999)
27. Veeman M. T., J. D. Axelrod & R. T. Moon: Functions and mechanisms of beta catenin-independent Wnt signaling. *Dev. Cell* 5, 367-377 (2003)
28. Giles R. H., J. H. Van Es & H. Clevers: Caught up in a Wnt storm: Wnt signaling in cancer. *Bioochim. Biophys. Acta* 1653, 1-24 (2003)
29. Amit S., A. Hatzubai, Y. Birman, J. S. Anderson, E. Ben Shushan, M. Mann, Y. Ben Nehriah & I. Alkalay: Axin-mediated CKI phosphorylation of beta-catenin phosphorylation at Ser 45; A molecular switch for the Wnt pathway. *Genes & Dev.* 16, 1066-1076 (2002)
30. Rubinfeld B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu & P. Polakis: Binding of GSK3 beta to the

APC-beta catenin complex and regulation of complex assembly. *Science* 272, 1023 – 1026 (1996)
31. Hart M., J. P. Concordet, I. Lassot, I. Albert, R. del los Santos, H. Durand, C. Perret, B. Rubinfeld, F. Margottin, R. Benarous & P. Polakis: The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. *Curr. Biol.* 9, 207-210 (1999)
32. Tamai K., X. Zeng, L. Chunming, Z. Xinjun, Y. Harada, Z. Chang & X. He: A mechanism for Wnt coreceptor activation. *Mol. Cell* 13, 149-156 (2004)
33. Tolwinski N. S., M. Wehrli, A. Rives, N. Erdeniz, S. DiNardo & E. Wieschaus: Wg/Wnt signal can be transmitted through arrow/LRP5,6 and axin independently of Zw3/Gsk3beta activity. *Dev. Cell* 4, 407-418 (2003)
34. Willert K., S. Shibamoto & R. Nusse: Wnt-induced dephosphorylation of Axin releases beta-catenin from the Axin complex. *Genes & Dev.* 13, 1768-1773 (1999)
35. Lustig B., B. Jerchow, M. Sachs, S. Weiler, T. Pietsch, U. Karsten, M. van de Wetering, H. Clevers, P. M. Schlag, W. Birchmeier & J. Behrens: Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol.* 22, 1184-1193 (2002)
36. Chia I. V. & F. Costantini: Mouse axin and axin2/conductin proteins are functionally equivalent *in vivo*. *Mol Cell Biol*. 25, 4371-4376 (2005)
37. Noordermeer J., J. Klingensmith, N. Perrimon & R. Nusse. Dishevelled and armadillo act in the wingless signalling pathway in Drosophila. *Nature* 367, 80-83 (1994)
38. Chen W., D. ten Berge, J. Brown, S. Ahn, L. A. Hu, W. E. Miller, M. G. Caron, L. S. Barak, R. Nusse & R.J. Lefkowitz: Dishevelled 2 recruits beta-arrestin 2 to mediate Wnt5A-stimulated endocytosis of Frizzled 4. *Science* 301, 1391-1394 (2003)
39. Umbhauer M., A. Djiane, C. Goisset, A. Penzo-Mendez, J. F. Riou, J. C. Boucat & D. L. Shi: The C-terminal cytoplasmic Lys-thr-X-X-X-Trp motif in frizzled receptors mediates Wnt/beta-catenin signalling. *EMBO J.* 19, 4944-4954 (2000)
40. Cliffe A., F. Hamada & M. Bienz: A role of Dishevelled in relocating Axin to the plasma membrane during Wingless signalling. *Curr. Biol*. 13, 960-966 (2003)
41. Kishida S., H.Yamamoto, S. Hino, S. Ikeda, M. Kishida & A. Kikuchi: DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. *Mol Cell Biol.* 19, 414-422 (1999)
42. Chen W., D. ten Berge, J. Brown, S. Ahn, L. A. Hu, W. E. Miller, M. G. Caron, L. S. Barak, R. Nusse & R.J. Lefkowitz: Dishevelled 2 recruits beta-arrestin 2 to mediate Wnt5A-stimulated endocytosis of rizzled 4. *Science* 301, 1391-1394 (2003)
43. Wong H. C., A. Bourdelas, A. Krauss, H. J. Lee, Y. Shao, D. Wu, M. Mlodzik, D. L. Shi & J. Zheng: Direct binding of of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Mol. Cell* 12, 1251-1260 (2003)
44. van Amerongen R., M. Nawijn, J. Franca-Koh, J. Zevenhoven, H. van der Gulden, J. Jonkers & A. Berns: Frat is dispensable for canonical Wnt signaling in mammals. *Genes & Dev.* 19, 425-430 (2005)
45. Molenaar M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree & H. Clevers: XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* 86, 391-399 (1996)
46. van de Wetering M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin & H. Clevers: Armadillo co activates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell*. 88, 789- 99 (1997)
47. Behrens J., J. P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl & W. Birchmeier: Functional interaction of β-catenin with the transcription factor Lef-1. *Nature* 382, 638-642 (1996)
48. Roose J., M. Molenaar, J. Peterson, J. Hurenkamp, H. Brantjes, P. Moerer, M. van de Wetering, O. Destree & H. Clevers: The Xenopus Wnt effector XTcf3 interacts with Groucho related transcriptional repressors. *Nature*, 395, 608-612 (1998)
49. Brantjes H., J. Roose, J., M. van De Wetering & H. Clevers: All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res. 29*, 1410-1419 (2001)
50. Daniels D. L. & W. I. Weis: Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat. Struct. Mol. Biol.* 12, 364-371 (2005)
51. Takemaru K. I. & R. T. Moon: The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J. Cell Biol*. 149, 249-254 (2000)
52. Barker N., A. Hurlstone, H. Musisi, A. Miles, M. Bienz & H. Clevers: The chromatin remodeling factor Brg-1 interacts with beta-catenin to promote target gene activation. *EMBO J.* 20, 4935-4943 (2001)
53. Kramps T., O. Peter, E. Brunner, D. Nellen, B. Froesch, S. Chatterjee, M. Murone, S. Zullig & K. Basler: Wnt/wingless signaling requires BCL9/Legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* 109, 47-60 (2002)
54. Thompson B., F. Townsley, R. Rosin-Arbesfeld, H. Musisi & M. Bienz: A new nuclear component of the Wnt signalling pathway. *Nat. Cell Biol.* 4, 367-373 (2002)
55. Townsley F. M., A. Cliffe & M. Bienz: Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. *Nature cell biology* 6, 626-633 (2004)
56. Bienz M. & H. Clevers: Armadillo/beta-catenin signals in the nucleus-proof beyond a reasonable doubt? *Nat Cell Biol*. 5, 179-182 (2003)
57. van de Wetering M., E. Sancho, C. Verweij, W. de Lau, I. Oving, A. Hurlstone, K. van der Horn, E. Batlle, D. Coudreuse, A-P. Haramis, M. Tjon-Pong-Fong, P. Moerer, M. van den Born, G. Soete, S. Pals, M. Eilers, R. Medema & H. Clevers. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 111, 241-250 (2002)
58. Pinto D., A. Gregorieff, H. Beghtel & H. Clevers: Canonical Wnt signals are essential for homeostasis of the intestinal epitelium. *Genes & Dev*. 17, 1709-1713 (2003)
59. B. Mao, W. Wu, Y. Li, D. Hoppe, P. Stannek, A. Glinka & C. Niehrs: LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* 411, 255-256 (2001)

60. Kuhnert F., C. R. Davis, H. T. Wang, P. Chu, M. Lee, J. Yuan, R. Nusse & C. J. Kuo: Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc. Natl. Acad. Sci*. 101, 266-271 (2004)
61. Ireland H., R. Kemp, C. Houghton, L. Howard, A. R. Clarke, O. J. Sansom & D. J. Winton: Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: Effect of loss of beta-catenin. *Gastroenterology* 126, 1236-1246 (2004)
62. van de Wetering M., I. Oving, V. Muncan, M. T. Pon Fong, H. Brantjes, H., D. van Leenen, F. C. Holstege, T. R. Brummelkamp, R. Ag ami & H. Clevers: Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO J. Rep*. 4, 609-615 (2003)
63. Kayahara T., M. Sawada, S. Takaishi, H. Fukui, H. Seno, H. Fukuzawa, K. Suzuki, H. Hiai, R. Kageyama H. Okano & T. Chiba: Candidate markers for stem and early progenitors, Musashi-1 and Hes1, are expressed in crypt base columnor cells of mouse small intestine. *FEBS Letters* 535, 131-135 (2003)
64. Okabe M., T. Imai, M. Kurusu & Y. Hiromi: Translational repression determines a neuronal potential in Drosophila asymmetric cell division. *Nature* 411, 94-98 (2001)
65. Potten C. S.: Stem cells in gastrointestinal epithelium; numbers, characteristics and death. *Philos. Trans. R. Soc. London B Biol. Sci* 353, 821-830 (1998)
66. Moser A. R., H. C. Pitot & W. F. Dove: A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 247, 322-324 (1990)
67. Luongo C., A. R. Moser, S. Gledhill & W. F. Dove: Loss of APC^{+} in intestinal adenomas from Min mice. *Cancer Res.* 54, 5947-5952 (1994)
68. Powell S. M., N. Zilz, Y. Beazer-Barclay, T. M. Bryan, S. R. Hamilton, S. N. Thibodeau, B. Vogelstein & K. W. Kinzler: APC mutations occur early during colorectal tumorigenesis. *Nature* (London) 359, 235-237 (1992)
69. Andreu P., S. Colnot, C. Godart, S. Gad, P. Chafey, M. Niwa-Kawakita, P. Laurent-Puig, A. Kahn, S. Robine, C. Perret & B. Romagnolo: Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the intestine. *Development and disease* 132, 1443-1451 (2005)
70. Grandori C., S. M. Cowley, L. P. James & R.N. Eisenman: The Myc/Max/Mad network and the transcriptional control of cell behaviour. *Annu. Rev. Cell Dev. Biol.* 16, 653-699 (2000)
71. He T-C., A. B. Sparks, C. Rago, H. Hermeking, L. Zawel, L. T. da Costa, P. J. Morin, B. Vogelstein & K. W. Kinzler: Identification of c-MYC as a target of the APC Pathway. *Science* 281, 1509-1512 (1998)
72. el-Deiry W. S., T. Tokino, T. Waldman, J. D. Oliner, V. E. Velculescu, M. Burrell, D. E. Hill, E. Healy, J. L. Rees & S. R. Hamilton: Topological control of p21 WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res*. 55, 2910-2919 (1995)
73. Zhu L. & A. Skoultchi: Coordinating cell proliferation and differentiation. *Curr. Opin. Genet. Dev.* 11, 91-97 (2001)
74. Quaroni A., J. Q. Tian & Ap. R.C. Seth: p27(kip) is an inducer of intestinal differentiation. *Am. J. Cell Physiol*. 279, C1045- C1057 (2000)
75. Deschenes C., A. Vezina, J. F. Beaulieu & N. Rivard: Role of p27(kip) in human intestinal differentiation. *Gastroenterology* 120, 423-438 (2001)
76. Nakayama K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, D. Y. Loh & K. Nakayama: Mice lacking p27 (kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85, 707-720 (1996)
77. Yang W. C., J. Mathew, A. Velcich, W. Edelman, R. Kucherlapati, M. Lipkin, K. Yang & L. H. Augenlicht: Targeted inactivation of the p21(waf1/cip1) gene enhances APC-initiated tumor formation and the tumor-promoting activity of a Western-style high risk diet by altering cell maturation in the intestinal mucosa. *Cancer Res*. 61, 565-569 (2001)
78. Zhang P., C. Wong, D. Liu, M. Finegold, J. W. Harper & S. J. Elledge: p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev*. 13, 213-224 (1999)
79. Batlle E., J. T. Henderson, H. Beghtel, M. M. van den Born, E. Sancho, G. Huls, J. Meeldijk, J. Robertson, M. van de Wetering, T. Pawson & H. Clevers: Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 111, 251-263 (2002)
80. van ES J. H., J. Philippe, A. Gregorieff, M. E. van Gijn, S. Jonkheer, P. Hatzis, A. Thiele, M. van den Born, H. Beghtel, T. Brabletz, M. M. Taketo & H. Clevers: Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nature Cell Biol.* 7, 381-386 (2005)
81. Ayabe T., D. P. Satchell, P. Pesendorfer, H. Tanabe, C. L. Wilson, S. J. Hagen & A. J. Ouellette: Activation of Paneth cell α -defensins in mouse small intestine. *J. Biol. Chem.* 277, 5219-5228 (2002)
82. Poliakov A., M. Cotrina & D. G. Wilkinson: Diverse roles of Eph receptors and Ephrins in the regulation of cell migration and tissue assembly. *Developmental cell* 7, 465-480 (2004)
83. Frisen J., J. Holmberg & M. Barbacid: Ephrins and their Eph receptors; multitalented directors of embryonic development. *EMBO J.* 18, 5159-5165 (1999)
84. Noren N. K. & E. B. Pasquale: Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins. *Cell. Signal*. 16, 655-666 (2004)
85. Wilkinson D. G.: Multiple roles of Eph receptors and ephrins in neural development. *Nat. Rev. Neurosci.* 2, 155-164 (2001)
86. Mellitzer G., Q. Xu & D. G. Wilkinson: Eph receptors and ephrins restrict cell intermingling and communication. *Nature* 400, 77-81 (1999)
87. Zorn A. M.: Wnt signalling: antagonistic Dickkopfs. *Curr. Biol.* 11, R592-595 (2001)
88. Lickert H., A. Kispert, S. Kutsch & R. Kemler: Expression patterns of Wnt genes in mouse gut development. *Mech. Dev*. 105, 181-184 (2001)
89. McBride H. J., B. Fatke & S. E. Fraser: Wnt signaling components in the chicken intestinal tract. *Dev. Biol*. 256, 18-33 (2003)

90. Gregorieff A., D. Pinto, H. Begthel, O. Destree, M. & H. Clevers. Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology*. 129, 626-638 (2005)
91. Shi Y. G. & J. Massague: Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113, 685-700 (2003)
92. Winesett M. P., G. W. Ramsey & J.A. Barnard: Type II TGF beta receptor expression in intestinal cell lines and the intestinal tract. *Carcinogenesis* 17, 989-995 (1996)
93. Barnard J. A., R. D. Beauchamp, R. J. Coffey & H. L. Moses: Regulation of intestinal epithelial-cell growth by transforming growth factor type-beta. *Proc. Natl. Acad. Sci. USA* 86, 1578-1582 (1989)
94. Markowitz S., J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler & B. Vogelstein: Inactivation of the type II TGFbeta receptor in colon-cancer cells with microsatellite instability. *Science* 268, 1336-1338 (1995)
95. Grady W. M., L. L. Myeroff, S. E. Swinler, A. Rajput, S. Thiagalingam, J. D. Lutterbaugh, A. Neumann, M. G. Brattain, J. Chang, S. J. Kim, K. W. Kinzler, B. Vogelstein, J. K. Willson & S. Markowitz: Mutational inactivation of transforming growth factor receptor type II in microsatellite stable colon cancers. *Cancer Res*. 59, 320-324 (1999)
96. Zhou S., P. Buckhaults, L. Zawel, F. Bunz, G. Riggins, J. L. Dain, S. E. Kern, K. W. Kinzler & B. Vogelstein: Targeted deletion of Smad4 shows it is required for transforming growth factor beta and activin signaling in colorectal cancer cells. *Proc. Natl. Acad. Sci. USA* 95, 2412-2416 (1998)
97. Sancho E., E. Batlle & H. Clevers: Signaling pathways in intestinal development and cancer. *Annu. Rev. Dev. Biol*. 20, 695-723 (2004)
98. Haramis A-P. G., H. Beghtel, M. van den Born, J. H. van Es, S. Jonkheer, G. J. A. Offerhaus & H. Clevers: De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* 303, 1684-1686 (2004)
99. Sayed M. G., A. F. Ahmed, J. R. Ringold, M. E. Anderson, J. L. Bair, F. A. Mitros, H. T. Lynch, S. T. Tinley, G. M. Petersen, F. M. Giardiello, B. Vogelstein & J. R. Howe: Germline SMAD4 or BMPR1A mutations and phenotype of juvenile polyposis. *Ann. Surg. Oncol*. 9, 901-906 (2002)
100. He Xi. C., J. Zhang, W-G. Tong, O. Tawfik, J. Ross, D. H. Scoville, T.Qiang, X. Zeng, X. He, L. M. Wiedemann, Y. Mishina & L. Linheng: BMP signaling inhibits intestinal stem cell self-renewal trough suppression of Wnt-beta-catenin signaling. *Nature Genet.* 36, 1117-1120 (2005)
101. Besson A., S. M. Robbin & V. W. Yong: PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis. *Eur. J. Biochem*. 263, 605-611 (1999)
102. Liaw D., D. J. Marsh, J. Li, P. L. Dahia, S. I. Wang, Z. Zheng, S. Bose, K. M. Call, H. C. Tsou, M. Peacocke, C. Eng & R. Parsons: Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat. Genet.* 16, 64-67 (1997)
103. Mutter G. L.: Pten, a protean tumor suppressor. *Am. J. Pathol*. 158, 1895-1898 (2001)
104. Persad S., A. A. Troussard, T. R. McPhee, D. J. Mulholland & S. Dedhar: Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. *J. Cell Biol.* 153, 1161-1174 (2001)
105. Groszer M., R. Ericksonm, D. D. Scripture-Adams, R. Lesche, A. Trumpp, J. A. Zack, H. I. Kornblum & X. WuH. Liu: Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science* 294, 2186-2189 (2001)
106. Kimura T., A. Suzuki, Y. Fujita, K. Yomogida, H. Lomeli, N. Asada, M. Ikeuchi, A. Nagy, T. W. Mak & T. Nakano: Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production. *Development* 130, 1691-1700 (2003)
107. Jamora C., R. DasGupta, P. Kocieniewski & E. Fuchs: Links between signal transduction, transcription and adhesion in epithelial bud development. *Nature* 422, 317-322 (2003)
108. Nybakken K. & N. Perrimon: Hedgehog signal transduction: recent findings. *Curr. Opin. Genet. Dev.* 12, 503-511 (2002)
109. Sukegawa A., T. Narita, T. Kameda, K. Saitoh, T. Nohno, H. Iba, S. Yasugi & K. Fukuda: The concentric structure of the developing gut is regulated by Sonic Hedgehog derived from endodermal epithelium. *Development* 127, 1971-1980 (2000)
110. Ramalho-Santos M., D. A. Melton & A. P. McMahon: Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 127, 2763-2772 (2000)
111. Mo R., J. H. Kim, J. Zhang, C. Chiang, C. C. Hui & P. C. Kim: Anorectal malformations caused by defects in sonic hedgehog signaling. *Am. J. Pathol*. 159, 765-774 (2001)
112. Kimmel S. G., R. Mo, C. C. Hui & P. C. Kim: New mouse models of congenital anorectal malformations. *J. Pediatr. Surg.* 35, 227-230 (2000)
113. van den Brink G. R., S. A. Bleuming, J. C. H. Hardwick, B. L. Schepman, G. J. Offerhaus, J. J. Keller, C. Nielsen, W. Gaffield, S. J. H. van Deventer, D. J. Roberts & M. P. Peppelenbosch: Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat Genet.* 36, 277-282 (2004)
114. Artavanis-Tsakonas S., M. D. Rand & R. J. Lake: Notch signaling; cell fate control and signal integration in development. *Science* 284, 770-776 (1999)
115. de Strooper B.: Aph-1, Pen-2, and Nicastrin with Presenillin generate an active gamma-Secretase complex. Neuron 38, 9-12 (2003)
116. Bray S. & M. Furriols: Notch pathway: making sense of suppressor of hairless. *Curr. Biol*. 11, R217-R 221 (2001)
117. S. Natalia & A. Gossler: Expression of Notch pathway components in fetal and adult mouse small intestine. *Gene expression patterns.* 2, 247-250 (2002)
118. Sander G. R. & B. C. Powell: Expression of notch receptors and ligands in the adult gut. *J. Histochem. Cytochem*. 52, 509-16 (2004)
119. Jarriault S., O. Le Bail, E. Hirsinger, O. Pourquie, F. Logeat, C. F. Strong, C. Brou, N. G. Seidah & I. A. Isra:

Delta-1 activation of Notch-1 signaling results in Hes-1 transactivation. *Mol. Cell Biol*. 18, 7423-7431 (1998)
120. Heitzler P., M. Bourouis, L. Ruel, C. Carteret & P. Simpson: Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signaling in Drosophila. *Development* 122, 161-171 (1996)
121. Yang Q., N. A. Bermingham, M. J. Finegold & H.Y. Zoghbi: Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* 294, 2155-2158 (2001)
122. Jenny M., C. Uhl, C. Roche, I. Duluc, V. Guillermin, F. Guillemot, J. Jensen, M. Kedinger & G. Gradwohl: Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. *EMBO. J*. 21, 6338-6347 (2002)
123. Jensen J., E. E. Pedersen, P. Galante, J. Hald, R. S. Heller, M. Ishibashi, R. Kageyama, F. Guillemot, P. Serup & O. D. Madsen: Control of endodermal endocrine development by Hes-1. *Nature Genet.* 24, 36-44 (2000)
124. Milano J., J. McKay, C. Dagenais, L. Foster-Brown, F. Pognan, R. Gadient, R. T. Jacobs, A. Zacco, B. Greenberg & P. J. Ciaccio: Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol. Sci*. 1, 341-358 (2004)
125. Crosnier C., N. Vargesson, S. Gschmeissner, L. Ariza-McNaughton, A. Morrison & J. Lewis: Delta-Notch signaling controls commitment to a secretory fate in zebrafish intestine. *Development* 132, 1093-1104 (2005)
126. van Es J., M. E. van Gijn, O. Riccio, M. van den Born, M. Vooys, H. Beghtel, M. Cozijnsen, S. Robine, D. J. Winton, F. Radtke & H. Clevers: Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Natur*e 435, 959-963 (2005)
127. Fre S., M. Huyghe, P. Mourikis, S. Robine, D. Louvard & S. Artavanis-Tsakonas: Notch signals control the fate of immature progenitor cells in the intestine. *Nature* 435, 964-968 (2005)
128. Capobianco A. J., P. Zagouras, C. M. Blaumueller, S. Artavanis-Tsakonas & J. M. Bishop: Neoplastic transformation by truncated alleles of human Notch1/Tan1 and Notch2. *Mol. Cell. Biol.* 17, 6265-6273 (1997)
129. Ohtsuka T., M. Ishibashi, G. Gradwohl, S. Nakanishi, F. Guillemot & R. Kageyama: Hes-1 and Hes-5 as Notch effectors in mammalian neuronal differentiation. *EMBO J.* 18, 2196-2207 (1999)
130. Galceran J., C. Sustmann, S-C. Hsu, S. Folberth & R. Grosschedl: Lef1-mediated regulation of Delta-like links Wnt and Notch signaling in somitogenesis. *Genes & Dev.* 18, 2718-2723 (2004)
131. Lynch H. T. & A. de la Chapelle: Hereditary colorectal cancer. *N. Engl. J. Med.* 348, 919-932 (2003)
132. Preston S. L., W. M. Wong, A. O. Chan, R. Poulsom, R. Jeffery, R. A. Goodlad, N. Mandir, G. Elia, M. Novelli, W. F. Bodmer, I. P. Tomlinson & N. A. Wright: Bottom-up histogenesis of colorectal adenomas: origin in the monocryptal adenoma and initial expansion by crypt fission. *Cancer Res*. 63, 3819-3825 (2003)
133. Shih I. M., T. L. Wang, G. Traverso, K. Romans, S. R. Hamilton, S. Ben-Sasson, K. W. Kinzler, K.W. & B. Vogelstein: Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci U S A*. 98, 2640-2645 (2001)
134. R. Fodde & P. M. Kahn: Genotype-phenotype correlations at the adenomatous polyposis coli (APC) gene. *Crit. Rev Oncog*. 6, 291-303 (1995)
135.Fodde R., W. Edelmann, K. Yang, C. van Leeuwen, C. Carlson, B. Renault, C. Breukel, E. Alt, M. Lipkin & P. M. Khan: A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors. *Proc. Natl. Acad. Sci. USA*. 91, 8969-8973 (1994)
136. Oshima H., M. Oshima, M. Kobayashi, M. Tsutsumi & M. M. Taketo: Morphological and molecular processes of polyp formation in APC(delta716) knockout mice. *Cancer Res.* 57, 1644-1649 (1997)
137. Quesada C. F., H. Kimata, M. Mori, M. Nishimura, T. Tsuneyoshi & S. Baba: Piroxicam and acarbose as chemopreventive agents for spontaneous intestinal adenomas in APC gene 1309 knockout mice. *Jpn. J. Cancer Res*. 89:392-396 (1998)
138. Sasai H., M. Masaki & K. Wakitani: Suppression of polypogenesis in a new mouse strain with truncated Apc(Delta 474) by a novel COX-2 inhibitor, JTE-522. *Carcinogenesis* 21, 953-958 (2000)
139. Rubinfeld B., B. Souza, I. Albert, O. Muller, S. H. Chamberlain, F. R. Masiarz, S. Munemitsu & P. Polakis: Association of the APC gene product with beta-catenin. *Science* 262, 1731- 1734 (1993)
140. Behrens J., B. A. Jerchow, M. Wurtele, J. Grimm, C. Asbrand, R. Wirtz, M. Kuhl, D. Wedlich & W. Birchmeier: Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 280, 596-599 (1998)
141. Zumbrunn J., K. Kinoshita, A. A. Hyman & I. S. Nathke: Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. *Curr. Biol.* 11, 44-49 (2001)
142. Berrueta L., J. S. Tirnauer, S. C. Schuyler, D. Pellman & B.E. Bierer: The APC-associated protein EB1 associates with components of the dynactin complex and cytoplasmic dynein intermediate chain. *Curr. Biol.* 9, 425-428 (1999)
143. Matsumine A., A. Ogai, T. Senda, N. Okumura, K. Satoh, G. H. Baeg, T. Kawahara, S. Kobayashi, M. Okada, K. Toyoshima & T. Akiyama: Binding of APC to the human homolog of the Drosophila discs large tumor suppressor protein. *Science* 272, 1020-1023 (1996)
144. Behrens J., B. A. Jerchov, M. Wurtele, J. Grimm, C. Asbrand, R. Wirtz, M. Kuhl, D. Wedlich & W. Birchmeier: Functional interaction of an Axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 280, 596-599 (1998)
145. Spink E. K., S. G. Fridman & W.I. Weis: Molecular mechanisms of beta-catenin recognition by adenomatous polyposis coli revealed by the structure of an APC-beta-catenin complex. *EMBO J.* 20, pp. 6203-6212 (2001)
146. Xing Y., W. K. Clements, I. Le Trong, T. R. Hinds, R. Stenkamp, D. Kimelman & W. Xu: Crystal structure of a beta-catenin/APC complex reveals a critical role for APC phosphorylation in APC function. *Mol Cell.* 15, 523-533 (2004)
147. Smits R., M. F. Kielman, C. Breukel, C. Zurcher, K. Neufeld, S. Jagmohan-Changur, N. Hofland, J. van Dijk,

R. White, W. Edelmann, R. Kucherlapati, P. M. Khan & R. Fodde: Apc1638T; a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes & Development* 13, 1309-1321 (1999)
148. Oshima M., H. Oshima, K. Kitagawa, M. Kobayashi, C. Itakura & M. Taketo: Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *Proc. Natl. Acad. Sci. USA*. 92, 4482-4486 (1995)
149. Albuquerque C., C. Breukel, R. van der Luijt, P. Fidalgo, P. Lage, F. J. Slors, C. N. Leitao, R. Fodde & R. Smits: The 'just-right' signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade. *Hum Mol Genet*. 11, 1549-60 (2002)
150. Henderson B. R.: Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat. Cell Biol*. 2, 653-660 (2000)
151. Rosin-Arbesfeld R., F. Townsley & M. Bienz: The APC tumour suppressor has a nuclear export function. *Nature* 406, 1009-1012 (2000)
152. Morin P. J., A. B. Sparks, V. Korinek, N. Barker & H. Clevers: Activation of beta-catenin-TCF signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275, 1787-1790 (1997)
153. Sparks A. B., P. J. Morin, B. Vogelstein & K. W. Kinzler: Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res*. 58:1130-1134 (1998)
154. Liu W., X. Dong, M. Mai, R. S. Seelan, K. Taniguchi, K. K. Krishnadath, K. C. Halling, J. M. Cunningham, L.A. Boardman, C. Qian, E. Christensen, S. S. Schmidt, P. C. Roche, D. I. Smith & S. N. Thibodeau: Mutations in Axin2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. *Nat. Genet*. 26, 146-147 (2000)
155. Batlle E., J. Bacani, H. Begthel, S. Jonkheer, A. Gregorieff, M. van de Born, N. Malats, E. Sancho, E. Boon, T. Pawson, S. Gallinger, S. Pals & H. Clevers: EphB receptor activity suppresses colorectal cancer progression. *Nature* 435, 1126-1130 (2005)
156. Jubb A. M., F. Zhong, S. Bheddah, H. I. Grabsch, G. D. Frantz, W. Mueller, V. Kavi, P. Quirke, P. Polakis & H. Koeppen: EphB2 is a prognostic factor in colorectal cancer. *Clin. Cancer Res*. 11, 5181-5187 (2005)
157. Lugli A., H. Spichtin, R. Maurer, M. Mirlacher, J. Kiefer, P. Huusko, D. Azorsa, L. Terraciano, G. Sauter, O. P. Kallioniemi, S. Mousses & L. Toernillo: EphB2 expression across 138 human tumor types in a tissue microarray: high levels of expression in gastrointestinal cancers. *Clin. Cancer Res*. 11, 6450-6458 (2005)
158. Huusko P., D. Ponciano-Jackson, M. Wolf, J. A. Kiefer, D. O. Azorsa, S. Tuzmen, D. Weaver, C. Robbins, T. Moses, M. Allinen, S. Hautaniemi, Y. Chen, A. Elkahloun, M. Basik, G. S. Bova, L. Bubendorf, A. Lugli, G. Sauter, J. Schleutker, H. Ozcelik, S. Elowe, T. Pawson, J. M. Trent, J. D. Carpten, O-P. Kallioniemi & S. Mousses: Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer. *Nature Genetics* 36, 979-983 (2004)
159. Kittles R. A., A. Boffoe-Bonnie, T. Moses, C. Robbins, C. Ahaghotu, P. Huusko, C. Pettaway C. S. Vijayakumar, J. Bennett, G. Hoke, T. Mason, S. Weinrich, J. Trent, F. Collins, S. Mousses, J. Bailey-Wilson, P. Furbert-Harris, G. Dunston, I. Powell & J. D. Carpten: A common nonsense mutation in EphB2 is associated with prostate cancer risk in African American men with a positive family history. *J. Med. Genet*. September (2005)
160. Oba S. M., Y. J. Wang, J. P. Song, Z. Y. Li, K. Kobayashi, S. Tsugane, G. S. Hamada, M. Tanaka & H. Sugimora: Genomic structure and loss of heterozygosity of EphB2 in colorectal cancer. *Cancer Lett.* 164, 97- 104 (2001)
161. Brabletz T., A. Jung, S. Spaderna, F. Hlubek & T. Kirchner: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat. Rev. Cancer*. 5, 744-749 (2005)

Abbreviations: (A-P) axis: anterior–posterior axis; APC: adenomatous polyposis coli; bHLH: basic Helix- Loop-Helix; BMP: bone morphogenetic protein; CKI: casein kinase I; CRC: colorectal cancer; DKK: Dickkopf; DN: dominant negative; Dsh: Dishevelled; Fabp: Fatty acid binding protein; FAP: familial adenomatous polyposis; FZD: Frizzled; GSK: glycogen synthase kinase; HES: hairy and enhancer of split; Hh: hedgehog; Hip: hedgehog interacting protein; HNPCC: Hereditary nonpolyposis colorectal cancer; Ihh: Indian hedgehog; LOH: loss of heterozygosity; LRP: Lipoprotein-related protein; MHAP: mixed polyposis syndrome; Min: multiple intestinal neoplasia; MMR: mismatch repair genes; NICD: Notch intracellular domain; PJ: Peutz-Jehgers syndrome; PTCH: patched; Shh: sonic Hedgehog; SMO: Smoothened; TA: transit amplifying; TGF: transforming growth factor; PI3K: phosphatidylinositol-3 kinase

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