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Major Signaling Pathways in Intestinal Stem Cells

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Abstract

Background—The discovery of markers to identify the intestinal stem cell population and the generation of powerful transgenic mouse models to study stem cell physiology have led to seminal discoveries in stem cell biology.

Scope of review—In this review we give an overview of the current knowledge in the field of intestinal stem cells (ISCs) highlighting the most recent progress on markers defining the ISC population and pathways governing intestinal stem cell maintenance and differentiation. Furthermore we review their interaction with other stem cell related pathways. Finally we give an overview of alteration of these pathways in human inflammatory gastrointestinal diseases.

Major conclusions—We highlight the complex network of interactions occurring among different pathways and put in perspective the many layers of regulation that occur in maintaining the intestinal homeostasis.

General significance—Understanding the involvement of ISCs in inflammatory diseases can potentially lead to new therapeutic approaches to treat inflammatory GI pathologies such as IBD and celiac disease and could reveal the molecular mechanisms leading to the pathogenesis of dysplasia and cancer in inflammatory chronic conditions.

Keywords

Intestinal Stem Cell; Wnt; Notch; Hedgehog; Bmp; Hippo; Inflammation

1 Introduction

The single cell-layer epithelium of the gastrointestinal (GI) tract represents the largest interface between the internal and external environment. The mucosa of the small intestine is composed of villi, finger-like protrusions that extend into the lumen of the gut, and

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intervillous regions that invaginate into the underlying lamina propria in the first postnatal weeks and form the crypts (Fig.1). The colon lacks villi and is only composed of crypts and surface epithelium. The tremendous cellular turnover capacity of the gut epithelium enables it to withstand alterations in the external milieu by renewing all the cells of the crypt-villus axis in 3 (mouse) to 7 (human) days [1, 2]. The epithelial cells are replenished continuously from a stem cell population in the crypt. These intestinal stem cells (ISC) give rise to the rapidly proliferating transit-amplifying cells that ultimately develop into absorptive enterocytes or secretory cells. The latter group can be further subdivided into mucus-secreting goblet cells, hormone-releasing enteroendocrine cells, antimicrobial Paneth cells, and the recently characterized chemo-sensing and opioid-secreting tuft cells [3, 4]. The differentiated post-mitotic cells migrate upwards to the villus and ultimately undergo anoikis when they lose cell-cell contact at the villus-tip. One exception to this rule is the Paneth cell lineage that moves downwards to the bottom of the crypts and is part of the intestinal stem cell niche [5].

In the small intestine, there are two putative distinct stem cell populations [6, 7]. Potten and coworkers identified a cell population situated at cell-position 4 starting from the bottom of the crypt, just above the Paneth cells, which is slowly dividing and retains markers like tritiated thymidine or bromodeoxyuridine (BrdU) that incorporate into the DNA during the S-phase [8]. They suggested that these '+4 cells' or label-retaining cells (LRC) represent the actual intestinal stem cell [9]. Recently, *Bmi-1*, *mTert* and *Hopx* were suggested as markers for these stem cells [10-12].

Around the same time, the crypt base columnar cell (CBC), a cell interspersed between the Paneth cells, was suggested by Cheng and Leblond as the intestinal stem cell [13]. *Lgr5* (Leucine-rich-repeat containing G-protein-coupled receptor 5 or *Gpr49*), a Wnt-target gene, was established as a solid marker for CBCs [14]. *Bmi1*-, *mTert*-, *Hopx*- and *Lgr5*-labeled cells were shown to give rise to the different intestinal cell lineages, i.e. multipotency, and to self-renew, thereby fulfilling the criteria for 'stemness' [10-12, 14]. This led to a model of two intestinal stem cell compartments with the actively cycling CBC that provide the progenitor cells on an everyday basis and the quiescent '+4 cells' that represent a reserve population that responds to injury like irradiation [6, 7, 15].

Recently, two groups independently showed a dynamic interplay between both cell populations [12, 15]. In an elegant study, Tian and colleagues demonstrated that *Lgr5*-positive cells are dispensable for the normal intestinal homeostasis and that an expanded *Bmi1*-positive population takes over its role after experimental ablation [15]. Moreover, in the recovery phase, the *Bmi1*-positive cells replenish the *Lgr5*-cell population. A similar interdependency between both populations was reported by Takeda et al [12]. Using *Hopx* as a marker for the '+4' cells, they found that they originate from or give rise to *Lgr5*-positive cells, suggesting a complicated bidirectional relationship [12]. Whether the '+4' and CBC ISCs truly represent different cell populations and, whether this is an intrinsic quality or is determined by their position in the niche, is still a matter of debate [16-18]. Interestingly, *Bmi1* and *mTert* label cells in the small intestine only, with a gradient from proximal to distal. Since the colonic architecture is preserved after *Lgr5* ablation, a similar quiescent population was also suspected in the colon [15]. Recently, *Lrig1*, a negative

regulator of ErbB signaling, was identified as a stem cell marker in the small intestine and colon, labeling a non-proliferating population at the bottom of the crypt that is distinct from *Lgr5*-positive cells [19]. This putatively novel stem cell population may represent a reserve stem cell in the colon although the interdependency between the *Lgr5* and the *Lrig1* population and the significance of the *Lrig1* cells during injury remain to be determined. Wong et al. confirmed the presence of *Lrig1* in the stem cell zone of the small intestine but contrary to Powell et al. a considerable overlap between the *Lgr5* positive CBCs and the *Lrig1* labeled cells was demonstrated [20].

The complicated architecture and cell composition of the different regions of the gastrointestinal tract is orchestrated by a complex interplay between the major signaling pathways. In this review we will focus on the adult mammalian small intestine, since this is the region that is most studied in stem cell biology and since its architecture with crypts and villi provides the unique opportunity to follow the entire lifetime of the epithelial cells in one axis: from the stem cells at the bottom of the crypt to the undifferentiated transit amplifying cells and finally the post-mitotic differentiated cells of the different lineages in the villus regions. For each pathway we will briefly describe the outline of the signaling pathway, followed by its role in self-renewal and/or differentiation and finally we will touch upon its role in non-neoplastic gastrointestinal disorders and the interaction with other stem cell related pathways.

2 Wnt

2.1 Overview of the pathway

Wnt-signaling is based on the autocrine and paracrine interaction of secreted cysteine-rich Wnt-glycoproteins with the seven-pass transmembrane receptor Frizzled (Fz) and the co-receptor lipoprotein-related protein (LRP). Binding of Wnt to its receptor activates the canonical pathway with stabilization and nuclear translocation of β -catenin or the non-canonical pathway which encompasses the Planar Cell Polarity and the Wnt/Ca(2+) pathway [21, 22]. The canonical pathway is the best characterized and the most relevant in stem cell signaling. Inactive β -catenin, the key mediator of the Wnt pathway, resides in the adherens junction or the cytoplasm and has a dual function in both cell adhesion and activation of Wnt-signaling. In the absence of binding of Wnt to its receptor, free cytosolic β -catenin is efficiently captured by the destruction complex, consisting of the scaffold protein Axin, adenomatous polyposis coli (APC), casein kinase1 (CK1), and glycogen synthase kinase 3 β (GSK3 β). These latter two kinases phosphorylate the amino-terminus of β -catenin which allows binding of an E3 ubiquitin ligase complex that targets β -catenin for proteosomal degradation. Upon binding of secreted Wnt-proteins to the Fz receptor, Disheveled (Dsh) proteins are phosphorylated causing the intracellular part of the co-receptor LRP to be phosphorylated by CK1 γ . This receptor complex sequesters axin which prevents proteosomal degradation and induces nuclear translocation of β -catenin. As a result, β -catenin interacts with T-cell factor/lymphoid enhancer factor (Tcf/Lef)-family members, displacing Groucho proteins that act as transcriptional repressors, and inducing Wnt-target gene transcription [23].

Nuclear localization of β -catenin, the hallmark sign of active Wnt-signaling, is localized to stem cells at the bottom of the intestinal crypts [24, 25]. Gregorieff et al. systematically analyzed the presence of several critical Wnt-pathway components in the proliferative compartment of the murine gastrointestinal tract by in-situ hybridization [26]. They demonstrated the presence of the Wnt agonists Wnt-3, Wnt-6b and Wnt-9b at the bottom of the crypt and co-staining of Wnt-3 and -9b with the Paneth cell marker lysozyme. The receptors Fz-5 and 7 and coreceptors, LRP-5 and -6, are expressed by the same cells, as opposed to Fz-6, which is expressed by cells along the crypt-villus axis except Paneth cells. The secreted Wnt-antagonists, Frizzled-related protein 1 and 5 (sFRP-1 and -5), compete with Fz-receptors, are present in the crypt mesenchyme, and are just above the Paneth cells respectively. Dickkopf 3 (Dkk-3), another secreted Wnt-inhibitor that binds to LRP co-receptors, was detected in villus mesenchyme [26]. Byun and colleagues demonstrated Dkk-2 and -3 in the colonic crypt epithelial cells [27]. In a dextran sodium sulphate (DSS)-colitis model, Dkk-1 was detected in colonic epithelial cells, lymphocytes, neutrophils and platelets, while in untreated mice, Dkk-1 was below the detection limit of fluorescence microscopy [28]. Tcf4, the major transcription factor of the canonical Wnt pathway in the gut, is expressed along the entire crypt-villus axis [26, 29]. Expression of Tcf4, however, does not imply active Wnt-signaling since Wnt-target gene transcription requires binding of β -catenin to, and displacement of, Groucho proteins from TCF4.

Together with the presence of essential components of the Wnt-signaling cascade in the proposed stem cell compartment, several groups have shown convincingly the importance of Wnt-signaling in proliferation and differentiation in the GI tract by knockout (KO) and transgenic animals. A recent discovery, however, was direct evidence of Wnt-activity in stem cells endowed with self-renewal and multi-potency [14]. The Wnt-target gene *Lgr5*, a seven span transmembrane G-protein coupled receptor, was expressed uniquely in the CBCs of the small intestine and also labeled stem cells in colon and stomach [14, 30]. Importantly, analysis of a *Lgr5*-EGFP-IRES-creERT2 mouse crossed with a Rosa26-LacZ reporter strain showed that these *Lgr5*-eGFP positive cells were multipotent and possessed the ability to self-renew indicated by fully labeled crypt-villus axes present up to 6 months [14, 31].

Recently three groups independently identified R-spondin as the endogenous ligand of the *Lgr5*- and the more widely expressed *Lgr4*-receptor [32-34]. R-spondins are secreted glycoproteins that amplify Wnt-signaling and induce a proliferative phenotype in vivo [35]. The expansion of organoid cultures is dependent on exogenous R-spondin1 [14, 32]. The endogenous source of R-spondin1 in the intestine is still unclear, although it has been located to enteroendocrine cells [35].

Paneth cells are established as an essential part of the stem cell niche in the small intestine by providing the Wnt agonist Wnt3 and the Notch ligand Dll4 to the adjacent CBCs [5]. Plating efficiency of *Lgr5* positive cells in organoid cell cultures was enhanced greatly in the presence of Paneth cells. Indeed, *Lgr5* positive CBCs maximize heterotypic contact with Paneth cells and avoid homotypic contact. The requirement of Paneth cells could be overcome by adding Wnt3A to the culture [5]. Recently, a c-Kit positive goblet cell subpopulation was identified in the colon that supports the CBCs in the colon, where Paneth cells are absent [36]. Co-culture of CBCs with these c-Kit positive cells improved colonoid

formation. The c-Kit positive cells expressed Dll1, Dll4 and epidermal growth factor. It is unclear whether these c-Kit CD44 positive cells also supply Wnt signals to the niche since the colonoids stopped growing and could not be passaged further without adding exogenous Wnt.

2.2 Wnt signaling is a 'conditio sine qua non' for maintenance of the stem-cell phenotype

The first suggestion of the importance of Wnt-signaling in intestinal stem cell regulation came from the observation of a mutation in the tumor-suppressor gene *APC* in familial adenomatous polyposis (FAP) patients, which was later shown to be one of the essential proteins involved in the canonical Wnt-pathway [37-39]. Since then, numerous reports implicated overactive Wnt-signaling as a key step in the formation of gastrointestinal adenomas and carcinomas, a topic beyond the scope of this review (recently reviewed in [40, 41]). The importance of the Wnt pathway on progenitor cells in the adult organism was demonstrated further using overexpression of Wnt-signaling such as the inducible knockouts for *Apc* (*Apc*^{-/-}) in mice [42, 43]. Within 5 days of *Apc* deletion, a considerable expansion of the proliferative progenitor compartment was observed with increased nuclear localization of β -catenin and expression of Wnt-target genes like *Lgr5*, *c-Myc* and *Cyclin-D1* [42, 43]. Importantly, deletion of *c-Myc* completely abrogated the phenotype associated with *Apc*^{-/-} [44]. Another more subtle overactivation of Wnt-signaling in a mouse model, with forced overexpression of an aminoterminal-truncated form of β -catenin that partially protects it from degradation, showed branched villi and an expanded proliferative compartment [45].

Several groups reported the loss of the proliferative compartment upon decreased Wnt-signaling. There was an absence of proliferating crypt cells in newborn *Tcf4* homozygous knockouts, leading to a decreased number of villi and neonatal lethality [46]. Similarly, intestine-specific ectopic overexpression of the Wnt inhibitor *Dkk1* converted the proliferating crypt cells into terminally differentiated enterocytes expressing alkaline phosphatase, a marker for differentiated absorptive enterocytes [25]. This correlated with the absence of the proliferation-inducing Wnt-target gene *c-Myc* and upregulation of the cell-cycle inhibitor *p21^{CIP1/WAF1}* [25]. Van de Wetering et al. showed that *c-Myc* overexpression could override the anti-proliferative effects of a truncated *Tcf4* by direct binding to the transcription activator MIZ-1 (synonym ZBTB17) in the promoter-region of *p21^{CIP1/WAF1}* halting its transcription [24]. Kuhnert et al. confirmed these data in a model with inducible overexpression of *Dkk1* delivered by an adenovirus vector in adult animals [47]. This resulted in 85% mortality within 10 days with associated weight loss and hematochezia. Microscopically, they observed a significant loss of crypts and villus blunting in the proximal small intestine. The loss of crypts occurred later in the colon, and was associated with the eventual development of colitis [47]. Interestingly, reduced *Dkk1* led to increased proliferation in the colon that was mediated by canonical Wnt-signaling. These data indicate that *Dkk1* has a basal function in attenuating crypt-cell proliferation in the colon [28]. Remarkably, the phenotype of the small intestine was unaltered by hypomorphic *Dkk1* [28].

c-Myc deletion can rescue the phenotype of Wnt-overactivation and overexpression of *c-Myc* can compensate for Wnt-hypoactivity [24, 44]. Compatible with this finding, intestine-

specific deletion of c-Myc in adult mice led to the progressive disappearance of the c-Myc deficient crypts and replacement by c-Myc proficient crypts over the course of 4 weeks through the process of crypt fissioning [48]. Bettess et al. reported that targeting *c-Myc* in the neonatal phase caused a transient decrease of the number of crypts [49]. However, the intestinal phenotype of intestine-specific deletion of *c-Myc* during adulthood was normal in the latter study with no histological differences between the c-Myc negative and the c-Myc positive crypts. The authors concluded that c-Myc is necessary during development, but redundant during adult life [49]. These data seemingly conflict with those of Muncan and colleagues in which c-Myc deficient crypts gradually disappeared after activation of the transgene [48]. The authors attributed these disparate findings to the use of a different promoter since villin is expressed more abundantly in the villi compared to the crypts, while *Cyp1A* that was used by Muncan et al. is more evenly expressed. Bettess et al. did not evaluate the progeny of the transgenic crypts by lineage tracing nor did they evaluate the evolution of the proportion of c-Myc deficient crypts over time which could indicate repopulation of c-Myc positive crypts through fissioning [49], compatible with the report by Muncan et al. [48]. Conversely, ectopic activation of *c-Myc* induces a dramatic increase in proliferative cells comparable to overactivity of Wnt by APC-inactivation [50].

Recently Klf4 emerged as a novel negative regulator of Wnt-signaling in the gastrointestinal tract [51]. Intestine-specific deletion of *Klf4* is characterized by increased proliferation, impaired goblet cell and absorptive enterocyte maturation and mislocalization of Paneth cells. This phenotype is reminiscent of other models of Wnt-hyperactivity, like APC-deletion. Conversely, overexpression of Klf4 leads to cell cycle arrest [52].

2.3 Role of Wnt signaling in differentiation

The current knowledge suggests that the effect of Wnt-signaling on differentiation is not an on-off phenomenon, that an appropriate amount of Wnt activity is necessary for orderly differentiation, and that deviations in both directions leads to impaired differentiation. In the presence of overactive Wnt-signaling, increased proliferation and maintenance of a cycling progenitor cell type impedes differentiation. Conversely, in the case of disrupted Wnt-signaling, progenitor cell depletion results in the absence of properly differentiated cells. Finally, there is a specific role for Wnt-signaling in Paneth cell differentiation.

Wnt-hyperactivity induced by deletion of *Apc* results in disturbed differentiation with absence of alkaline phosphatase expression, a maturation marker for absorptive enterocytes, loss or drastic reduction of goblet cells, and decrease in enteroendocrine cells [42, 43]. Similar to the changes in proliferation induced by APC-deficiency, this perturbed differentiation is dependent on *c-Myc* expression and can be rescued by *c-Myc* deletion [44]. The ectopic expression of c-Myc also induces loss of goblet cells [50]. These data highlight the conflicting duality of proliferation vs. differentiation.

In models of decreased Wnt signaling, similar differentiation abnormalities are observed. In neonatal *Tcf4*^{-/-} mice, enteroendocrine cells are absent, but goblet cells develop normally [46]. Paneth cells cannot be assessed at such an early time point since they develop only in the second postnatal week. Pinto and colleagues showed a complete absence of all secretory

cells in a mouse model with ectopic overexpression of *Dkk1* [25], which correlated with the absence of the secretory cell master transcription factor, *Atoh1*.

Models of increased and abrogated Wnt signaling showed convincingly that the development of Paneth cells is directly dependent on Wnt-signaling. *Cryptdin*- and *defensin*-genes, two Paneth cell markers, are direct targets of Wnt-signaling [43]. The number of Paneth-like cells (lysozyme positive but lacking typical granules) are increased in APC-deficiency, but distributed along the crypt-villus axis suggesting migration defects [42, 43]. Moreover, Paneth cells are detectable in the colon, an area normally devoid of Paneth cells, in APC-deficient mice. A similar phenotype results from expression of β -catenin resistant to GSK-3 β phosphorylation [53]. Conversely, Paneth cell differentiation was impaired in animals expressing a hypomorphic β -catenin, suggesting that the development of Paneth cells directly depends on Wnt-signals [53]. This was further supported by a significant downregulation of Paneth cells after deletion of β -catenin in Neurogenin- (*Ngn3*) positive secretory cell precursors. *Ngn3* is a basic helix-loop-helix (bHLH) protein that has a well-established role in the generation of enteroendocrine cells in the gut and pancreas [54] and is positioned downstream of the Notch-Hes1-*Atoh1* cascade (cf. Notch signaling). Lineage-tracing of *Ngn3*-descendants label all enteroendocrine cells, but also 5-10% of goblet cells and 45% of Paneth cells [54]. Deletion of β -catenin in *Ngn3*-positive cells did not affect the number of enteroendocrine or goblet cells, suggesting that their maturation is Wnt-independent [55]. In contrast, there was an 80% reduction of the *Ngn3*-dependent Paneth cells indicating that a major fraction of the Paneth cells depend on Wnt signals for their development.

Sox9, a high-mobility group box transcription factor that is expressed in the intestinal stem cell region, is critical for Paneth cell differentiation as demonstrated by the complete absence of Paneth cells in mice deficient for *Sox9* [5, 56, 57]. *Sox9* is positioned downstream of Wnt signaling and is not dependent on *c-Myc* [44, 58]. Interestingly, the *Sox9*-deficient crypts were enlarged, harbored more proliferative cells and an increased amount of cells positive for Musashi-1, a putative intestinal stem-cell marker [56, 57]. The authors speculated that this could be explained by a negative feedback loop of SOX9 which suppresses the transcriptional activity of β -catenin [58, 59]. These findings, however, contrast with a recent report by Sato et al. [5]. In an inducible mouse model of *Sox9*^{-/-}, they observed a progressive loss of Paneth cells and reduction of CBCs over the course of 8 weeks after induction of the transgene. Eventually, all *Sox9* deficient crypts were replaced by the neighboring *Sox9* wild-type crypts by crypt fission. The reason for the different conclusion of Sato et al. vs. Bastide et al. and Mori-Akyama et al. is unclear. The latter two studies used a non-inducible conditional KO under the villin-promoter, which is active from E10.5 [60], while Sato's study used an inducible KO model. The different role of *Sox9* during development vs. homeostasis of the adult intestine may account for the disparate conclusions. The role of Sox-factors in stem cell function is complicated and recent data nicely show that *Sox9* has a dose-dependent inverse relationship with proliferation in the crypt base. Cells sorted by low expression of *Sox9* (*Sox9*^{low}) are capable of self-renewal, are multipotent and form organoids in vitro which establishes them as stem cells [58, 61, 62].

Reducing, but not abolishing, *Sox9* expression (e.g. through incomplete KO) could lead to expansion of the proliferative zone in this model.

2.4 Appropriate Wnt-activity is indispensable for normal cell migration through Eph/Ephrin expression

Migration in the gastrointestinal tract and other organ systems is tightly orchestrated by the cell-cell contact dependent EphB/EphrinB-signaling through their remodeling effect on the actin cytoskeleton [63-65]. EphB2 and EphB3 are tyrosine kinase receptors that are positively controlled by Wnt-activity as opposed to their ligand EphrinB1 that is repressed by Wnt-signaling [24]. This dependency on Wnt-signaling is reflected in the localization of the different components of the Ephrin signaling cascade. EphB3 is highly expressed in the CBCs and Paneth cells, while EphB2 follows a decreasing gradient from the bottom of the crypt to the transit amplifying zone. Conversely, the ligands Ephrin-B1 and -B2 are highly expressed at the crypt-villus junction with a decreasing gradient to the bottom of the crypt [63, 66]. Interaction between Eph and Ephrin results in repulsion between both cells, thereby establishing a boundary between proliferative and differentiated cells. In the absence of EphB3, Paneth cells are displaced from their normal position and distributed randomly, suggesting that their normal downward migration depends on the presence of EphB3 [67]. Remarkably, the phenotype of conditional deletion *Fz5* is indistinguishable from *EphB3*^{-/-}, indicating that the EphB3-regulated migration occurs via Fz5 [67]. Holmberg and colleagues confirmed and extended these data by demonstrating a reduction of the proliferative compartment after blocking EphB/EphrinB signaling [66]. These data were corroborated by showing expansion of the proliferative compartment in response to expression of a constitutively active EphB receptor. They explained these data resulted from a direct influence of EphB-signaling on proliferation [66]. Interestingly, proliferation is also increased moderately in mice expressing a constitutively active (ligand-independent) EphB2, in which the Paneth cell localization was unaltered [66].

2.5 Involvement of Wnt in non-neoplastic gastrointestinal disorders

The mutation of the *APC* tumor-suppressor gene in FAP patients is the most illustrative example of the contribution of overactive Wnt-signaling in colorectal cancers. Mutations in *APC* or other components of the Wnt-pathway are present virtually in every patient with colorectal carcinoma. Remarkably, Wnt-activity in cancer is also regulated to some extent, suggesting that an optimal balance of Wnt is required for malignant transformation as postulated in the 'just right' hypothesis [68, 69]. Discussion of cancer stem cells, however, is beyond the scope of this report (recently reviewed in [40, 41]).

There is accumulating evidence showing that alterations in Wnt-signaling play a role in the pathophysiology of chronic inflammatory bowel disease (IBD). Paneth cells express NOD2, which is an intracellular receptor for muramyl dipeptide (MDP), a bacterial peptidoglycan [70]. There was a lower production of Paneth cell derived α -defensins in ileal Crohn's disease (CD), which was more pronounced in patients carrying a *NOD2(CARD15)*-mutation, one of the major genes involved in the heritability of CD [71]. Wehkamp et al. showed that decreased expression of TCF-4 in ileal biopsies of CD correlated with the expression of α -defensins and did not depend on the level of inflammation or the *NOD2*-genotype [70]. In a

multi-center follow-up study a single nucleotide polymorphism (SNP) in the *TCF4* gene was associated with ileal CD, but not with colonic CD or ulcerative colitis (UC) [72].

It is well-known that chronic inflammatory conditions like IBD confer an increased risk for the development of gastrointestinal carcinoma [73], yet there is only limited insight regarding the interaction between gastrointestinal inflammation and the stem-cell niche. Recently, Dahan et al. showed that lamina propria lymphocytes of active CD and UC in co-culture with intestinal cell lines induced activation of the PI3/Akt- and MAPK- pathway [74]. Moreover, there was a significantly higher expression of p-Akt and p-MAPK in IBD intestinal epithelial cells [74]. In two different experimental models of colitis (infectious and IL10-deficiency) the PI3/Akt pathway was identified as a molecular link between inflammation and Wnt-signaling [75, 76]. Treatment with a PI3K-inhibitor decreased activation of Wnt-target genes like *cyclin-D1* and *c-Myc* and subsequent epithelial proliferation [75, 76]. Bacterial clearance was impaired by blocking PI3K without influencing the adaptive immune response [76]. The PI3K/Akt pathway thus represents a new potential therapeutic target for IBD and IBD-related cancer [77].

3 Notch

3.1 Overview of the Notch pathway

Notch-signaling is based on direct contact between adjacent cells bearing Notch-ligands and –receptors [78-80]. The initiating step of the Notch-signaling cascade is the interaction between one of the five single-pass trans-membrane ligands Delta-like (Dll) 1, 3 and 4, Jagged 1 and 2, and the single-pass trans-membrane Notch-receptors Notch 1-4 [81, 82]. Upon binding of the ligand, a conformational change of the receptor is followed by two proteolytic cleavages. In the first step, the ectodomain is shed by an ADAM-metalloprotease and subsequently, the receptor is cleaved by a presenelin-dependent γ -secretase resulting in the release and nuclear translocation of the Notch intracellular domain (NICD) [83]. NICD forms a complex with the transcription factor CSL (RBP-J κ in mice) that turns the CSL-complex from a transcriptional suppressor into an activator of transcription of Notch-target genes [78, 82, 84]. This pathway is referred to as the canonical Notch pathway [78].

In recent years evidence has accumulated that Notch-signaling has a critical function in intestinal stem cell function. Indirect support came from studies analyzing localization of the expression of the key components of the Notch-signaling cascade and the consequences of constitutional and conditional knockouts. *Hes1* (Hairy enhancer of split), the major Notch-target gene, is expressed in the epithelial cells at the bottom of the crypt and co-localizes with the putative stem-cell marker Musashi-1 (Msi1) in the both the CBCs and the +4-cells [85, 86]. Notch1 and -2 were shown to be the only Notch-receptors expressed in the intestinal crypts, contrary to Notch3 and -4 that are present in the intestinal mesenchyme and endothelium [87-89]. Expression of Notch1 mRNA is enriched in CBCs, although some Notch1 expression is detectable also in post-mitotic villus cells [84, 90].

Jagged 1, Jagged 2, Dll1 and Dll4 were identified as the potential Notch-ligands in the gut [88, 91-94]. *In situ* hybridization showed the presence of *Dll1*, *Dll4* and *Jagged1*-mRNA in the crypts [88]. At the protein level, Dll4 was detectable in all secretory cells and Jagged 1

and 2 were demonstrated in a few scattered cells [95]. It is unknown, however, whether Jagged 1 has a physiological role in the gut since combined *Jagged1* – *Dll1* knockouts do not differ from *Dll1* single knockouts [94].

Recently, several groups convincingly demonstrated active Notch-signaling in intestinal stem cells by lineage tracing studies. Vooijs *et al.* engineered the NIP-Cre mouse that is a cross between a R26R reporter mouse and a strain in which the intracellular domain of Notch1 was replaced by a Cre-recombinase [84]. This innovative genetic construct allowed monitoring of the descendants of cells in which active Notch1 signaling occurred at any stage of their lifetime [84]. Uniformly labeled, monoclonal crypts were visualized feeding cells to polyclonal villi, suggesting that there is active Notch1 signaling in stem cells. Moreover, cells of the four lineages were positive for β -galactosidase indicating multipotency of the Notch1-active cells. This was confirmed in a tamoxifen-inducible strain of NIP-Cre [94]. Eight months after a single injection of tamoxifen, whole crypt-villus structures were labeled, indicating that the cells with active Notch1 signaling are capable of self-renewal and multipotency, the prerequisites for stemness. Interestingly, the *Notch1* lineage follows a gradient from the proximal to distal intestinal tract, with few labeled cells in the colon [84, 87, 94], which may be due to a slower division rate of colonic stem cells [14]. Similar experiments evaluating all 4 Notch receptors demonstrated that only Notch1, and to a much lesser extent Notch2, were active in intestinal stem cells [87]. Notch1 was present in both the '+4' cells (60%) and CBCs (30%) [87], however, the '+4' stem cell zone was defined by cell-position only. This location is prone to overestimation since only a small fraction of all the cells in the '+4' ring represents true stem cells. Further studies are warranted for validated markers for the '+4'-cells, like Bmi, mTert and Hopx.

3.2 Notch-signaling preserves stemness

Several groups demonstrated the activity of Notch in CBC stem cells, but until recently it was unknown whether Notch-signaling was necessary and/or sufficient to preserve the intestinal stem cell phenotype as in other stem cell systems [82, 96]. Transgenic models with deletion or overexpression of the different ligands, receptors, and downstream targets of Notch-signaling, identified Notch as a crucial player in the preservation of stemness of the gastrointestinal stem cell compartment. In *Drosophila*, however, inhibition of Notch leads to expansion of intestinal stem cells, contrary to the role of Notch in self-renewal in mammalian model systems [80, 97].

Intestine-specific deletion of *Notch1* or the lower expressed *Notch2* does not lead to an intestinal phenotype [89], although others have shown a mild intestinal phenotype in Notch1-deficient animals [84, 98]. Transgenic crypts in the aforementioned NIP-Cre mouse were characterized by a modest increase in the goblet cell lineage [84] and antibody-mediated blocking of Notch1 increased the number of goblet cells and reduced the number of proliferative cells [98]. Blocking both Notch1 and Notch2 led to conversion of proliferative cells to postmitotic goblet cells, phenocopying Rbp-J deficiency [89, 94]. These data suggest that both Notch receptors have overlapping actions, but are not completely redundant. Partial redundancy is also present in the Notch-ligands since targeting of the individual ligands *Dll1*, *Dll4* and *Jagged1* did not result in a reduction of the proliferative

compartment, although *Dll1* knockout moderately increased goblet and enteroendocrine cells [94, 95]. A combined *Dll1-Dll4* knockout and Rbp-J deficiency led to the loss of expression of the CBC-markers *Olfm4*, *Lgr5* and *Ascl2* and correlated with a complete disappearance of Ki67 positive cells in the crypt [94]. Paneth cells, a putatively important part of the stem cell niche, express Dll4 to maintain the adjacent CBC in an undifferentiated state [5]. The role of Notch in preserving stemness was confirmed by studies showing expansion of the proliferative compartment and absence of differentiation in mice carrying the constitutively active Notch1 receptor (expression of NICD) under the villin promoter [99]. Ultrastructurally, the cells resemble immature progenitor cells with fewer and more fragile microvilli leading to malabsorption and death within 3 days after birth [99].

Several mechanisms are proposed by which Notch-signaling maintains the progenitor state. It was suggested that Hes1 binds the promoter of the cyclin-dependent kinase inhibitors p27^{Kip1} and p57^{Kip2}, halting their transcription and keeping the cell in an undifferentiated, proliferating state [89]. Others showed, however, that at least part of the proliferative capacity of Notch-signaling is mediated via downregulation of Atonal homolog1 (Atoh1, Math1 or HATH1) [100, 101]. Atoh1 is a bHLH transcription factor that is suppressed by Hes1, possibly by direct interaction with the *Atoh1* promoter [100, 102-105]. The proliferative compartment was preserved upon Notch inhibition by γ -secretase inhibitor (GSI) treatment in Atoh1-negative crypts in an intestine-specific mosaic knockout for *Atoh1* [100]. These findings indicate that Hes1-mediated repression of p27^{Kip1} and p57^{Kip2} is dispensable to preserving the ability of stem cells to self-renew. The data are corroborated by the observation that intestine-specific knockouts of Atoh1 have higher levels of proliferation and lower expression of the cell-cycle inhibitor p27^{Kip1}, which could not be restored by Notch inhibition by GSI [100, 101]. Similarly, the proliferative compartment was preserved and even expanded slightly in a combined *Rbp-J* and *Atoh1* knockout, although the authors attributed this change to a complex and Notch-independent regulation of Hes1 since the dual knockout had normal Hes1-levels [106]. A central role for Atoh1 in regulation of proliferation is consistent with its identification as a tumor suppressor gene in mouse models of colon cancer and human tumors [101, 107, 108].

Van Dussen et al. provided supportive evidence in an inverse genetic model in which *Atoh1* was expressed constitutively under the villin-promoter [109]. This genotype induces lethality in the perinatal period and featured blunted villi and a smaller proliferative compartment. The transcription of the CBC-marker *Lgr5* was decreased significantly, suggesting differentiation of CBCs. *Prominin1*-expression, which is a putative marker for both CBCs and transit-amplifying cells, was upregulated in the *Atoh1*-transgenic animals and interpreted by the authors as a shift from pluripotent stem cells toward transit-amplifying cells [109-111]. Together, these data support a model in which Notch-signaling preserves self-renewal in the intestinal progenitor cells by suppressing Atoh1.

This conclusion was challenged recently in a paper by the same group in which they showed that *in vivo* Notch inhibition by GSI induced a substantial decrease in GFP-labeled cells in *Lgr5*-GFP mice [110]. Moreover, *in vitro* pretreatment of isolated *Lgr5*-GFP positive cells with a GSI decreased efficiency of organoid cultures [110]. Mechanistically, the loss of stem cells was linked to the loss of olfactomedin4 (*Olfm4*) expression, one of the putative CBC

stem cell factors [112]. *Olfm4* is a Notch target gene and is regulated independently from *Atoh1* as *Olfm4* was downregulated after GSI in an *Atoh1*-deficient fetal intestine organ culture [110], suggesting that the Notch-mediated preservation of self-renewal is an *Atoh1*-independent phenomenon. Unfortunately, there was no direct assessment of the proliferative compartment in *Atoh1*-deficient GSI-treated organ cultures. Moreover, the function of *Olfm4* is still unknown and it is a matter of debate whether it contributes to stem cell function since *Olfm4*-deficient intestines are reported to have a normal phenotype [113].

3.3 Notch-signaling induces differentiation towards the absorptive enterocyte lineage

Notch-signaling holds a central position in the cell-fate decision of secretory vs. non-secretory lineage development. The first evidence of the primary importance of Notch in intestinal cell differentiation was provided by Jensen *et al.* who showed excessive numbers of the secretory lineage cells in *Hes1*-deficient mice [114]. Subsequently others confirmed that inhibition of Notch-signaling at different levels of the signaling cascade induced goblet cell metaplasia [89, 94, 98, 102, 110]. Several groups showed that secretory cell differentiation by Notch inhibition was completely dependent on the upregulation of the bHLH-transcription factor *Atoh1* [106, 110]. *Atoh1* is epistatic to Notch-signaling in secretory cell specification, since a combined inducible knockout of *Rbp-J* and *Atoh1* was a phenocopy of *Atoh1* single knockout characterized by a complete absence of secretory cells [89, 98, 103, 106, 114-117]. Interestingly, the morphology and expression of differentiation markers of absorptive enterocytes was normal in a combined inducible knockout of *Rbp-J κ* and *Atoh1*, highlighting again the central role of *Atoh1* [106].

Considerable controversy remains regarding the effects of Notch-signaling on non-goblet secretory cells like Paneth cells and enteroendocrine cells with reports indicating no change [94, 102] and increases in enteroendocrine cells [100, 106, 114, 117] or Paneth cells [100, 106, 110, 114, 118] after Notch-inhibition. These differences are attributed perhaps to the timing of the analysis relative to gene targeting since Paneth cells have a longer lifespan compared to absorptive enterocytes and goblet cells [119]. Another possibility is the lack of full maturation of Paneth cells. An *Atoh1*-dependent increase in expression of markers of the four secretory cell lineages was documented after GSI treatment. Paneth cells, however, did not attain an adult phenotype and were positive for both goblet cell and Paneth cell markers. Remarkably, Paneth cells express Notch-ligands like *Dll4*, but are not known to receive Notch signals [5, 110]. Since Paneth cells are a proposed part of the stem cell niche [5], this impaired Paneth-cell maturation could contribute to a decreased stem cell function.

One of the major transcription factors implicated in late goblet cell differentiation is Kruppel-like factor 4 (*Klf4*). Homozygous deletion of *Klf4* induces neonatal lethality and causes near-absence of mature goblet cells in the colon on postnatal day 1 [120]. Several groups demonstrated that Notch-inhibition by GSI led to increased *Klf4*-expressing cells in small intestinal and colonic crypts [121, 122]. Elements responsive to the activated form of Notch1 are identified in the *Klf4*-promoter leading to repression of transcription [122]. These findings were challenged in an inducible intestine-specific *Klf4* knockout model in which no differences in goblet cells were observed 2 weeks after tamoxifen-induction [94]. Moreover, the phenotype of *Rbp-J – Klf4 vil-Cre-ERT2* double deficient mice was

indistinguishable from *Rbp-J^{vil-Cre}-ERT2* animals [94]. These disparate results may be attributed to the age of genetic targeting and organ-specific changes (colon vs. small intestine). This was recently confirmed by Ghaleb et al. who demonstrated that deletion of *Klf4* had a more profound impact on goblet cell differentiation in the colon compared to the small intestine [51]. Intriguingly, *Klf4* deletion also conferred increased Wnt-signaling (cf. section on Wnt-signaling).

Notch also plays a role in the regulation of the numbers of goblet cells relative to absorptive enterocytes. Until recently it was unclear why there was a natural preponderance of absorptive enterocytes to secretory cells in a 13:1 ratio in the small intestine, since a stochastic model of lateral inhibition would lead to a 3:1 ratio [123]. It was found that soon after a transient acquisition of Dll1, the secretory precursor loses its ability to divide, contrary to the cells receiving the Notch-signal from these secretory cells by lateral inhibition [95]. These absorptive precursors divide further until exiting the crypt progenitor niche thus leading to a natural dominance of absorptive enterocytes. Others, however, challenged this view by showing that some secretory progenitors co-expressed the proliferation marker Ki67 [103].

3.4 Involvement of Notch in non-neoplastic gastrointestinal disorders

Contrary to the numerous reports on involvement of Notch-signaling in GI cancer models, the data on involvement in non-neoplastic diseases in the GI tract are scarce. Recently Notch was implicated in IBD. Several groups reported an increase in proliferation and loss of goblet cells in active ulcerative colitis which correlated to increased expression of NICD of Notch1 and Hes1 [105, 124-126]. Okamoto et al. suggested Notch is implicated in the regenerative response of the epithelium since there was increased pathology, weight loss and mortality in a murine DSS-colitis model when the Notch pathway was inhibited by GSI treatment in the late phase of colitis [124]. On the other hand, if Notch was impaired in an early stage of DSS-colitis the damage was reduced, probably by preventing the loss of goblet cells, which exert a protective role in the initial phase [127]. Of interest was the report of increased Notch-1 signaling in inflamed vs. non-inflamed CD, but no changes in UC [125]. Likewise Dll-4 and Hes1 were upregulated in CD, but not in UC. Surprisingly, there was a positive correlation between Hes1 and Atoh1-expression in CD [125].

Capuano et al. found reduced goblet cells and lower Notch1- and *Klf4*-expression in pediatric celiac disease patients, which was controlled by increased microRNA-449a [128]. These data, however, are inconsistent with animal modeling data in which decreased Notch-signaling impairs proliferation and increases goblet cell differentiation. Goblet cell function, epithelial integrity and intestinal regeneration are of major importance in inflammatory disorders of the intestine. Clearly, more research is necessary on the role of Notch, one of the master regulators of the proliferation-differentiation switch, in inflammatory intestinal pathologies.

4 Hedgehog

4.1 Overview of the Hedgehog pathway

The Hedgehog (Hh) proteins are part of a family of secreted proteins involved in development of many organs including neural tube, axial skeleton, limbs [129], lung, skin, and gastrointestinal tract [130, 131]. Hh proteins are necessary for development and play an important role in maintaining cellular homeostasis in various tissues, including the GI tract. Hh is synthesized as an inactive 45kD precursor. The Hh precursor undergoes post-translational modification and autocleavage [132]. The Hedgehog N-terminal domain mediates the signaling activity. It undergoes post-translational modifications like covalent coupling with cholesterol and palmitoylation, which are critical in enhancing Hh membrane association, secretion, and range of action of the active fragment [133]. The C-terminus is required to promote autocatalytic cleavage. Hh was first discovered in *Drosophila* as a central regulator of patterning and polarity of the larval segmentation [134]. *Drosophila* has only one gene coding for Hh, mammals, including humans, possess three *Hh* genes: Indian Hedgehog (*Ihh*), Desert Hedgehog (*Dhh*) and Sonic Hedgehog (*Shh*). Zebrafish has a fourth Hh components named *tiggywinkle hedgehog*. Although they are expressed in specific domains during development and adult life [131, 135], they are interchangeable and have similar affinity for their receptors. Other components of the Hh pathway were identified first in *Drosophila* with homologues in vertebrates such as Dispatched, which is needed to release the mature Hh protein; the 12 transmembrane receptor Patched (Ptc) in *Drosophila* that is PTCH1 and -2 in vertebrates; the seven transmembrane protein Smoothened (SMO); the transcription factor Cubitus interruptus in *Drosophila* that corresponds to three proteins GLI1,2,3 in vertebrates. Other key components of the Hh pathway, dispensable or absent in *Drosophila*, but required in vertebrates, are the inhibitor HH interacting protein (HHIP) that binds to HH competing with PTCH and suppressor of fused (SUFU), a cytoplasmic protein involved in sequestering the inactive forms of the transcription factors GLI. Other accessory proteins identified in vertebrates include KIF (the homolog of *Drosophila* Cos-2) and FU that directs GLI towards proteasome mediated degradation. Another relevant difference in Hh signaling in vertebrates, is the requirement of the primary cilium. Although the primary cilium is absent mostly from *Drosophila* cells and dispensable in Hh pathway [136], it is present virtually in all mammalian cells. Indeed, the integrity of the primary cilium is mandatory for intact Hh pathway and studies showed that many human ciliopathies are related to alterations in the Hh pathway.

Since the discovery of Hh, a number of studies have elucidated the factors involved in its regulation and mechanism of action. A detailed description of the complex signaling cascades in Hh pathway is beyond the scope of this review but was the focus of several recent excellent reviews [137, 138]. Briefly, in the absence of HH, the receptor PTCH interacts, either directly or indirectly, with SMO and inhibits its function. This repression, mediated by SUFU, results in Ci/GLI transcription factor functioning as a transcriptional repressor. When HH binds to PTCH, it relieves repression on SMO allowing active SMO to drive the conversion of the GLI transcription factors from their inactive to active state so they can enter the nucleus and transcribe HH target genes. Activation of the Hh pathway

also upregulates expression of members of its own transduction cascade such as *Ptch1* and the inhibitor *Hhip*.

4.2 Role of Hh in the intestinal stem cell compartment

HH proteins are expressed in specific regions of the GI tract where they interact with other pathways that regulate development [130, 139] and homeostasis of the gut. Recent studies show that in adults, *Hh* is expressed exclusively in the epithelium, while the target cells that express the receptor *Ptch* and downstream components of the signaling pathway reside in the underlying mesenchyme [130, 139-141]. *Shh* and *Dhh* are expressed mainly in the stomach. *Shh* is also expressed at very low levels by a few epithelial cells residing at the bottom of the small intestinal and colon crypts and in the cecum [142, 143]. *Ihh* is expressed all along the GI tract [144, 145]. In the intestinal epithelium, *Ihh*, is expressed at the base of the villi [146, 147] and at reduced levels at the tip of the villus [141, 145]. In the colon, *Ihh* is expressed in the fully differentiated enterocytes at the top of the crypts [148].

Hh knockouts have severe developmental defects and are embryonic lethal. To investigate the activity of this pathway in the adult GI tract, a number of conditional transgenic mice were generated to target Hh or other components of the pathway. The generated mutants differ in terms of phenotype penetrance depending on the targeted gene and time of the conditional activation. In the small intestinal epithelium of conditional *Ihh* KO [*villin-Cre; Ihh^{flox/flox}*] mice and in transgenic mice expressing a truncated soluble form of the Hh inhibitor protein *Hhip* [12.4*villin-flox-lacZ-flox-Hhip* TM; *villin-Cre* also referred as *VFHhip*] and [12.4*villin-Hhip* TM, also referred as *villin-Hhip* TM], there was an overproliferation of the intestinal epithelium with lengthening and fissioning of crypts and an expansion of the Wnt signal responsive compartment [140, 141, 149]. Generation of ectopic crypts along the villus axis was observed only in the more severe mutants [140, 141]. Similarly, in colon treated with cyclopamine, an alkaloid inhibitor of Hh signaling through its ability to bind SMO, there was a decrease in the differentiation of the colonic epithelium and increase in Wnt pathway activity [148]. As expected, constitutive activation of Hh in the colonic epithelium through conditional deletion of *Ptch* [*Ptch^{flox/flox}; CreERT2*], resulted in crypt hypoplasia, and premature enterocyte differentiation with induction of the BMP pathway and inhibition of Wnt pathway [150]. Of interest is that overexpression of *Ihh* in the small intestine had no effect on the proliferative zone [149]. Further investigation is needed to ascertain if the contrasting data on the outcome of HH loss in colon versus small intestine reflect regional differences in the modulation of stem cell populations or are due to the different transgenic model systems used. Studies of Hh on cell lineage differentiation in colon in *Ihh* KO mouse revealed an expansion of the secretory cell lineage at the expense of absorptive cells. In addition, the absorptive cells did not fully differentiate into mature enterocytes [140, 141]. These findings were not observed in other mouse models where Hh was only mildly reduced [149]

At the molecular level, microarray analysis of colon tissue with ablated *Hh* signaling [*Villin-Cre;Ihh^{flox/flox}*] showed upregulation of Wnt target genes such as *c-Myc*, *Sox-9*, *Lgr5*. Relative to other signaling pathways involved in maintaining the stem cell compartment, *Bmp4* and respective downstream transcription factors, *Id1*, *Id2* and *Id4*, were reduced

significantly. No differences were found in Notch and Ras/MAPK pathways [140]. Thus, disruption of Hh pathway causes overproliferation of the ISC compartment and reduction of absorptive cell lineage differentiation in both small intestine and colon. In the small intestine, lengthening and fissioning of crypts and expanded Wnt signal responsive compartment, generation of ectopic crypts along the villus axis were also reported. Microarray data suggest that Hh downregulation reflects on BMP pathway reduction and upregulation of Wnt pathway with no effect on Notch.

4.3 Hh pathway alteration affects mesenchyme organization

The intestinal subepithelial myofibroblasts (ISEMF) and muscularis mucosae are in close association with the epithelium in the pericrypt region and along with Paneth cells are thought to represent the ISC niche [5]. These mesenchymal cells secrete various factors like cytokines, extracellular matrix proteins [151] and growth factors such as BMP antagonists like gremlin [152] that modulate the ISC compartment. Recent studies suggest that HH exerts an indirect effect on the epithelial ISC compartment by interfering with maturation and localization of the underlying stromal cells that in turns generate signaling molecules needed for the maintenance of the ISC niche. Downregulation of the Hh pathway in the mesenchyme of the small intestine or colon results in a mislocalization and reduction of ISEMF [140, 141], reduction of mature smooth muscle cells (SMC) from intravillus region and muscularis mucosae and accumulation of immature smooth muscle precursors [140, 141, 147, 149]. Conversely, in a model system where Hh is upregulated, there was an accumulation of myofibroblasts [150] and expansion of SMC in the villus core [147, 149].

Microarray analyses conducted on both colon and small intestine samples with reduced HH activity confirm these histological findings. The genes most affected by the decreased HH activity were several metalloproteases (MMPs) that were upregulated [140, 149] and genes involved in smooth muscle development (*Myh10*, *Myh11*, *myocardin*, *desmin*), extracellular matrix (*laminin*, *fibronectin*), and integrins were downregulated [140].

Both myofibroblasts and mature SMC require Hh signaling for proliferation and differentiation [140, 153]. Most of the evidence points to BMP4 as a downstream pathway in the implementation of Hh signaling; however, there is evidence that at least smooth muscle cell precursors can respond directly to HH stimulation in promoting differentiation into SMC. Stimulation of the murine embryonic mesenchymal cell line, C3H10T1/2, with HH, when the pan BMP inhibitor Noggin was simultaneously administered, resulted in differentiation into mature smooth muscle cells. In particular, the activation of myocardin, a master regulator of SMC differentiation, occurred with kinetics similar to other HH target genes both *in vivo* and *in vitro*. Sequence analysis of the *myocardin* promoter confirmed the presence of potential GLI1 binding sites [149]. These observations, taken together, suggest that HH regulation of *myocardin* may occur directly through GLI1 bypassing the BMP pathway.

In conclusion, loss of Hh signal causes reduction and mislocalization of myofibroblasts and SMC and accumulation of immature smooth muscle cell precursors. In addition, there is evidence of direct activity of Hh pathway in driving smooth muscle differentiation.

4.4 Involvement of Hh in non-neoplastic gastrointestinal disorders

The Hh pathway is implicated in T lymphocyte development [154, 155] and activation of CD4⁺ T cells and macrophages [156]. Zacharias *et al.* showed in *VFHhip* transgenic mice that a reduced Hh signal resulted in the influx of fibroblasts and macrophages into the villus core indicative of activation of an innate inflammatory response in the small intestine [157]. Similar studies demonstrated that a chronic reduction in *Ihh* resulted in progressive leukocyte infiltration of the crypt area, blunting or loss of villi, and development of intestinal fibrosis [158]. Microarray expression data generated from mesenchyme deprived of epithelial Hh signals revealed increased expression of several chemokines, metalloproteases, and proinflammatory cytokines supporting the involvement of Hh pathway in modulating the pro-inflammatory response [157]. The infiltrated CD3⁺ T lymphocytes and CD19⁺ B cells did not respond to HH. A subpopulation of CD11b⁺ and CD11c⁺ cells resembling dendritic cells, however, expressed the Hh target transcription factor *Gli1* and showed reduced IL-6 release when treated with HH ligands [157], supporting the idea that Hh may act as a tolerogenic signal in the intestine. In contrast, upregulation of components of the Hh pathway was reported in inflammatory diseases of the GI tract, including IBD, Barrett's esophagus, and chronic gastritis [159, 160]. In IBD, *Shh* and *Ptch1* were highly expressed in intraepithelial and mucosal inflammatory cells [159]. These apparently contradictory data may be due to tissue-specific expression of *Shh* versus *Ihh*, and to the fact that the human disease samples analyzed often depict a more complex process than that observed in controlled transgenic model systems. More experiments are needed to ascertain if the observed differences between mice and humans reflect divergence in the function of the Hh pathway.

In humans, *GLII*, an effector of HH activity is located within the IBD2 linkage region 12q13.3 [161]. This observation prompted an epidemiologic study to search for SNPs associated with *GLII* gene in three independent Caucasian populations of IBD patients (5000 people). It was found that an SNP in *GLII* open reading frame occurred in a significant number of ulcerative colitis patients in two of the three populations that were screened. The identified SNP resulted in a missense mutation leading to the conversion of a glutamine to a glutamic Acid (Q1100E). Functional analysis of mutated *Gli1* showed reduced trans-activation ability in a luciferase reporter assay, confirming the reduced functionality of this GLI1 isoform. Finally, transgenic mice *Gli1*^{+/-LacZ}, heterozygous for functional *Gli1* were more prone to develop DSS-induced colitis than the control littermates, supporting the concept that *Gli1* can be haploinsufficient [153].

This study suggests that intact Hedgehog signaling is critical to the ability of the gut to respond to pro-inflammatory stimuli and that reduced GLI1 function is implicated in the development of UC/IBD. These data were challenged recently by another study that evaluated a broader population. Bentley and colleagues showed that the identified *GLII* SNP does not increase susceptibility to IBD across all the Caucasian populations, but it also does not exclude a contribution to UC onset in the populations studied by Lees *et al.* [162].

Taken together these studies indicate that *Ihh* is a critical sensor of epithelial integrity. It promotes the maturation of cells residing in the underlying mesenchyme which then signal

back to the epithelium. Consequently, the effect of Hh on the ISC compartment is indirect and mediated by the BMP4 pathway (see next section). Hh also promotes maturation of tolerogenic immune cells in the small intestine. Disruption in the Hh pathway may contribute to the development of innate immune response and to the pathogenesis of autoimmune diseases such as UC. The link between Hh pathway modulation of inflammation and stem cell proliferation remains an area of active investigation.

5 Bmp

5.1 Overview of the BMP pathway

Bone morphogenetic proteins (BMPs) are soluble factors belonging to the TGF- β cytokine family. The first description of BMPs was based on their role in regulating limb development and cartilage formation [163, 164]. More recently, they were shown to be involved in several developmental processes as well as in adult tissue homeostasis [103, 165]. There are ten members in this family of proteins in humans, most of which share primary sequence homology, the exception being BMP1, which is a metalloprotease.

Depending on the specific cellular context, BMPs regulate differentiation, apoptosis and cell growth. BMPs undergo multiple levels of regulation ensuring a tight control of their activity. They are secreted in their active form [166] and homodimerize before binding to their cognate receptors (BMPR). Their activity is regulated by soluble antagonists in the extracellular space, which sequester them from binding to their receptors. There are many inhibitors of BMP that are classified based on homology including Follistatin, Noggin, Chordin/SOG and DAN/Cerberus families [167].

BMPs bind to a class of Ser/Thr receptor (R) type I/II heterodimers. There are four type I [Alk1, Alk2, Alk3 (BMPR1A), Alk6 (BMPR1B)] and three type II (BMPR-II, ActR-IIA and ActR-IIB) receptors (R) identified. BMP2, -4 and -7 specifically activate Alk3 (BMPR1A) and Alk6 (BMPR1B) type I receptors, while BMP6 and BMP7 (but not BMP2 or -4) bind the ALK2 receptor [168, 169]. The primary sequence of BMPRs contains an N-terminal extracellular binding domain, a transmembrane domain and a Ser/Thr kinase C-terminal domain. The type I receptor has a domain consisting of three GlySer (GS domain) repeats in proximity of the kinase domain. Binding of the BMPs occurs first to the type I receptor, then the complex BMP/Type I receptor binds with high affinity to the type II receptor. The proximity of type I/II receptors allows the phosphorylation of the GS domain by type II constitutive kinase domain and converts the type I receptor into its active form.

The BMP/BMPR activated complex signals through the phosphorylation of members of the family of proteins SMAD, the homologues of *Drosophila* gene *Mothers against decapentaplegic* (*Mad*). SMAD proteins include three classes of proteins: Receptor associated Smad (R-SMAD), Cooperating Smad (Co-SMAD) and Inhibitory Smads (I-SMAD). R-SMAD1, -5, and -8, bind to the active BMPR and exclusively transduce BMP signaling, while R-SMAD2, -3 belong to the TGF- β /Activin signaling pathways. The co-Smad, SMAD4, and I-Smads, SMAD6, -7, are common components of all BMPR. Phosphorylated R-SMADs form heteromeric complexes with Co-SMAD4. The active Smad complex then translocates efficiently to the nucleus where, along with other transcription

factors, activates the expression of genes specifically regulated by BMPs. SMAD6 inhibits BMP signaling by sequestering SMAD1 in an inactive SMAD1-6 complex [170], whereas SMAD7 can bind directly to the activated BMP type I receptor, competing with R-Smads.

BMP specific target genes include the inhibitor *Smad6* and *Smurf1*. SMURF1 is an E3 ubiquitin ligase that selectively promotes poly-ubiquitination of SMAD1, -5 and targets them for proteasome-mediated degradation. This mechanism ensures the termination of BMP signal in un-stimulated cells. Another class of genes directly under the control of BMPs is the *Id1-4* family (inhibitor of differentiation or inhibitor of DNA binding proteins). IDs contain a bHLH dimerization motif, but lack the DNA binding domain. They act as dominant negative regulators of transcription factors containing bHLH motifs by forming dimers with bHLH-containing transcription factors and thus preventing their binding to DNA or co-transcription factors. Many bHLH transcription factors positively regulate cell differentiation while ID proteins act as positive regulators of cell proliferation and negative regulators of cell differentiation. Specific examples of transcription factors inhibited by IDs include MyoD [171] and E1A [172] and components of the cell cycle machinery, such as Rb [173]. BMPs can also inhibit proliferation in neural stem cells [174] and hematopoietic stem cells [175]. The repressive actions of BMPs on proliferation can also occur through cell cycle downregulation by upregulating and/or improving the stability of p21 cyclin-dependent kinase inhibitor [176, 177].

5.2 BMPs in preservation of 'stemness' and differentiation of intestinal progenitor cells

The expression of BMP pathway components in the intestine is complex. A systematic study of gene expression in the small intestinal epithelium versus mesenchyme of perinatal mouse showed that *Bmp4*, -5, -6 are expressed almost exclusively in the mesenchyme, while *Bmp1*, -2 and -7 are expressed in both areas. The receptors: *Bmpr-II*, *Alk4*, *Alk2* (*ACVR1*), *Bmpr1A* and intracellular components of the pathway *Smad1*, -5, -6, and 7, are expressed in both epithelium and mesenchyme. Finally, BMP inhibitors including the three follistatin family members (*follistatin*, *follistatin 5*, and *follistatin-like 1*), *chordin-like1*, and *gremlin1* are expressed primarily or exclusively in the mesenchyme [178]. Similar studies in the colon showed that *Bmp2*, *Bmpr1A*, *Bmpr1B*, *Bmpr-II*, *Smad1*, and *Smad4* are found predominantly in mature colonocytes at the epithelial surface [179]. BMP inhibitors like *gremlin1*, 2 and *chordin-like1* are expressed by the mesenchymal cells surrounding the colon crypts, which are primarily myofibroblasts and smooth muscle cells [152].

Studies making use of transgenic knockdown mice have shed light on the function and mechanism by which BMP signaling is implemented by both mesenchyme and epithelium to control the intestinal stem cell proliferation.

In experimental models the reduction of BMP signal in both epithelium and mesenchyme by knocking down the receptor *Bmpr1A* or by overexpressing the BMP inhibitor *noggin* [180] resulted in hyperproliferation of the intestinal stem cells, increased number of crypts, ectopic crypts along the crypt/villus axis, and later development of intestinal polyposis [180, 181]. Interestingly, an epithelial cell specific knockout of *Bmpr1A* showed a less penetrant crypt fission phenotype and elongated villi, but an absence of *de novo* crypt formation and polyps even in one year old animals [182]. These data suggest that BMPs function as a quiescence

signal, restraining fissioning of crypts by inhibiting intestinal stem cell proliferation. Finally the immune cells have also a prominent role in controlling the ISC proliferation as proposed by a study of a selective knock out in T cells of *Smad 4*, part of the BMPs signaling, that led to epithelial tumors throughout the gastrointestinal tract, although its epithelial-specific deletion did not [183].

BMPs might control ISC acting both directly and indirectly. BMPs can, in fact, prevent stem cell proliferation by antagonizing Wnt pathway in the epithelial stem cells via activation of PTEN and consequent inactivation of Akt mediated accumulation of β -catenin into the nucleus [184].

Another BMP-mediated mechanism to control stem cell regulation is suggested by studies in cultured colon cancer cells, in which BMPs exert a negative control on cell cycle progression by stabilizing the cyclin-dependent kinase inhibitor p21^{CIP/WAF} [177]. These data are in agreement with the observation that BMP inhibitors are expressed and secreted by cells surrounding the intestinal crypts [152], ensuring a BMP-free ISC niche. The mild increased proliferation observed in the *Bmpr1A* epithelial knock out, however, suggests that a direct role of BMP on the epithelial stem cells is not predominant. The emergent working model in these studies is that the cells residing in the intestinal mucosa, such as mesenchymal cells, are likely the main target of BMP signaling, which, in turn, downregulates epithelial proliferation.

Interestingly BMP has a direct role in the differentiation of the epithelium. Knocking down the BMP receptor *Bmpr1A* in the epithelium resulted in an inefficient differentiation of the secretory/neuroendocrine lineage [182], with no change in the numbers of goblet and Paneth cells, but impaired (Paneth and goblet) differentiation based on presence of fewer secretory granules and reduced expression of maturation markers. The absorptive cell lineage was unaffected, but the number of enteroendocrine cells was reduced significantly.

The role of ID1-4 BMP effectors in the intestine remains unclear. ID1-4 act predominantly to prevent cell differentiation and promote cell proliferation in many cell types, yet there is no clear evidence of their role in intestinal stem cell regulation. Real-time PCR data demonstrated that ID proteins are expressed in mouse intestinal mucosa and respond to perturbations in BMP signaling [150]. *Id2* and *Id3* expression increases in cells that exit the cell cycle approaching the crypt-villus junction [185]. In addition, *Id3* is highly expressed in ISC compartment [186]. In the small intestine, ID targets, bHLH transcription factors, are required for the development of the secretory lineage. Mutations in *Atoh1* [103] resulted in a loss of all three secretory lineages, while mutation in a downstream bHLH transcription factor, *neurogenin3* (*Ngn3*), affected only enteroendocrine development [54, 187]. Reduction in BMPs signaling and corresponding reduction in Ids, would be expected then to result in upregulation of bHLH transcription factors and eventually in expansion of the secretory lineage. Unexpectedly, this did not occur and there was actually a reduced differentiation and maturation of the secretory lineage [182]. Similarly, overexpression of *Id1* in the small intestinal epithelium did not affect the secretory cell lineage [185]. Other evidence supports the concept that IDs can promote differentiation instead of proliferation in the small intestine. It has been shown that ID2 drives differentiation and acts as a tumor

suppressor in small intestine [188] and epigenetic silencing of *Id4* was reported in gastric adenocarcinoma [189].

In conclusion, BMP signaling is critical in adult intestinal homeostasis by preventing over-proliferation of the intestinal stem cell compartment. Although both epithelium and underlining mesenchyme express the BMP signal transduction components, the evidence suggests that the mesenchyme is the main target of BMPs. Specialized mesenchymal cells, in turn provide the optimal niche for the ISC proliferation by expressing BMP antagonists while outside the crypts they restrain ISC proliferation.

Finally, BMP signaling is required for proliferation, terminal differentiation and maturation of intestinal cell precursors of the secretory lineage, particularly the neuroendocrine cells, whereas they do not affect the absorptive lineage of the small intestine. The BMPs effectors, ID proteins, are involved in sustaining both proliferation and differentiation. Their dual apparently contradictory activity may be modulated by the specific milieu of the cell expressing them. Further studies could reveal novel aspects of ISC maintenance and lineage commitment by BMPs in the small intestine.

5.3 Involvement of Bmp in non-neoplastic gastrointestinal disorders

The large family of Bmp proteins is involved in proliferation and differentiation of stem and progenitor cells of multiple tissues with an even more prominent role in the process of bone and cartilage development. For this reason most of the BMP therapeutics under investigation are designed to overcome defects in bone and cartilage development and repair (reviewed in [190]). The involvement of this pathway in stem cell biology has prompted interest in its role in the development and metastasis of different cancers. In the gastrointestinal tract *BMPRIA* and *SMAD4*, both part of the BMP4 pathway, were found mutated in patients with Juvenile polyposis syndrome [191-193]. Research is currently focused on its involvement in tumor progression and metastasis of colon cancer (reviewed by [194]) and there are no data that associate defects in BMP pathways with other intestinal diseases.

6 Hippo/YAP

6.1 Overview of the Hippo/YAP pathway

The Hippo signaling pathway has been implicated only recently in mammalian stem cell biology and the different components of the signaling cascade are still being identified. The core pathway consists of a kinase cassette in which the complex formed by MST1/2 and WW45/SAV1 phosphorylates LATS1/2 and MOB1. Subsequently, the central effector molecules of the cascade, YAP or TAZ, are phosphorylated which creates a binding site for 14-3-3 proteins, leading to nuclear exclusion and inactivation of YAP and TAZ (Reviewed in [195]). There is scant information about the upstream components in the mammalian pathway. Most of the *Drosophila* proteins have mammalian counterparts, but few are implicated definitively in the mammalian Hippo cascade. Nuclear YAP and TAZ interact with several transcription factors of which members of the TEAD/TEF family (TEAD1-4) seem to represent the main mediators of YAP/TAZ-induced transcription [195].

Yap1 is expressed in the crypts of the small intestine [196-199]. In the colon, Yap1 expression is more abundant and was shown in the cytoplasm of both proliferating and post-mitotic cells [198, 199]. The upstream negative regulator MST1 is localized in the cytoplasm of the cells in the proliferative compartment and in the nucleus of differentiated cells [200]. Together these data indicate that the Hippo-pathway is active in post-mitotic, differentiated intestinal epithelial cells leading to nuclear exclusion of YAP. In the absence of active Hippo-signaling, MST1 resides in the cytoplasmic compartment and unphosphorylated YAP accumulates in the nucleus inducing gene-transcription.

In a murine model of inducible *Yap1* overexpression, the proliferative zone in the intestine was expanded greatly with Ki67 positive-cells over the entire crypt-villus axis [196]. The cells had a progenitor phenotype with fewer and fragile microvilli and death occurred within a few days after induction. The proliferative phenotype was reversible upon withdrawal of induction of the transgene [196]. A similar proliferative phenotype was reported in *Sav1*-deficient and *Mst1/Mst2* double knockout animals [198-200], although no lethality was reported in intestine-specific *Sav1*-knockouts [198]. The embryonic lethality of a constitutive *Sav1*-deficiency is most likely due to extra-intestinal effects of the genotype since placental malformation was observed [200]. A similar difference in lethality was observed in constitutional vs. conditional combined knockouts for *Mst1* and *Mst2*, although the latter also succumbed at the age of 13 weeks [199]. Ablation of *Yap* rescues the phenotype of combined *Mst1/Mst2* deficiency, confirming that YAP is positioned downstream of these kinases [199].

Differentiation of secretory cells was impaired in all models of overactive YAP or knockouts of proteins of the core kinase cassette [196, 199, 200]. Interestingly, in a model of intestine-specific *Yap*-deletion, small intestinal and colonic histology was indistinguishable from wild-type littermates [198, 199]. Together these data suggest that YAP is dispensable for normal homeostasis, but that Hippo signaling is critical to prevent YAP overactivity, which leads to uncontrolled proliferation without differentiation.

6.2 Involvement of Hippo/YAP in non-neoplastic gastrointestinal disorders

The Hippo signaling cascade has not been implicated thus far in non-neoplastic gastrointestinal disease; however, there is evidence that decreased Hippo-signaling is involved in the regeneration process after intestinal injury [198]. Cai et al., showed increased expression of *Yap* and a decreased phospho-YAP/YAP ratio in the regeneration phase after DSS colitis. Consistent with a role for Hippo in intestinal repair, they observed increased histological damage and lethality in DSS-treated *Yap*-deficient mice [198]. This group also reported development of polyps in *Sav*-deficient intestines, which closely resembled sessile serrated polyps (SSP) in humans and up-regulation of nuclear YAP in human SSP in the absence of nuclear β -catenin which is present in classic adenomas [198].

7 Interactions among stem cell pathways

The complex balance between maintaining the progenitor cell-type in the crypts and achieving adequate transition to maturation and differentiation once the progenitor cells move up the crypt-villus axis requires a complex interaction between the different pathways.

The major interactions are schematically shown in figure 2. Both Wnt and Notch are of major importance in the maintenance of the stem cell phenotype. The capacity of Notch-signaling to preserve self-renewal in progenitor cells is dependent on the presence of intact Wnt-signaling. The expanded proliferative compartment in mice with a constitutively active Notch1-receptor is not observed in a compound mutant mouse that is also deficient in Tcf4, the main transcription factor of the Wnt signaling cascade in the gut [201]. Moreover, when Notch is activated in the villus region, where Wnt-signaling is inactive, this does not induce a major proliferative response in the villus, contrary to the crypt region with high Wnt-activity [99, 202].

Many studies on the interaction of Wnt and Notch have been performed in human and murine colorectal carcinoma models, an area beyond the scope of this review. We will briefly summarize the most important other findings. In the presence of overactive Wnt-signaling like in *Apc*^{-/-} mice, GSI-treatment halts proliferation in adenomas [102]. Contrary to these findings, Peignon *et al.* showed that an inducible *Apc* homozygous KO and a combined *Apc* and *Rbp-J* KO were phenotypically identical [34]. This might be due to downregulated Atoh1-levels by Wnt-signaling. They also demonstrated that β -catenin, the central effector in the canonical Wnt-pathway, induced expression of *Hes1* by direct binding to the *Hes1* promoter, positioning Notch downstream of canonical Wnt-signaling [108]. The fact that *Hes1* expression is preserved in *Apc-Rbp-J* double KO supports the complex regulation of *Hes1* by Notch and Wnt-signaling, at least in *Apc*-mutated animals. The effects of Notch on differentiation are reported to occur independently from Wnt [201]. Taken together these data support the following model. Cells not subjected to Notch-activation undergo differentiation to a secretory phenotype, which represents the default cell type of the intestinal epithelium. Conversely, cells that receive active Notch-signaling from their neighboring cell in a process called lateral inhibition, will develop towards an absorptive phenotype or continue to self-renew in the Wnt-dependent stem-cell compartment.

The Bmp-pathway inhibits proliferation in the intestinal progenitor compartment by antagonism of Wnt-signaling [203]. Inactivation of the Bmp-receptor BMPRI_A expands the proliferative compartment in the crypt, suggesting a homeostatic function of Bmp in keeping self-renewal within limits, i.e. within the stem-cell niche [181]. The most likely mode of interaction occurs via the PTEN/PI3K pathway. When phosphatidylinositol 3-kinase (PI3K) is activated by binding of a ligand to a receptor tyrosine kinase, it will phosphorylate phosphatidylinositol-4,5-diphosphate (PIP₂) to PI-3,4,5-triphosphate (PIP₃). This eventually leads to the recruitment and phosphorylation of Akt, which is the main effector kinase of this pathway [204]. P-Akt can phosphorylate β -catenin at the Ser552-position, promoting its nuclear activity [181]. Another interaction between canonical Wnt and PI3K/Akt may exist via Akt-mediated inhibition of GSK3 β , leading to enhanced Wnt-signaling, although this latter mechanism is still controversial [77]. The levels of p-Akt are tightly controlled by the negative regulator, unphosphorylated phosphatase and tensin homologue (PTEN) which will dephosphorylate PIP₃ to PIP₂. Active Bmp-signaling will lead to the activation (i.e. dephosphorylation) of PTEN [181]. The influence of Akt on Wnt-signaling has been complicated recently by the report of increased nuclear export of β -catenin upon Akt-induced phosphorylation of the nuclear shuttle protein Chibby [205].

The effects of Hh on the ISC compartment are indirect and occur mainly through regulation of the BMP pathway. Many BMPs are expressed in both small intestine and colon (see BMP section), however data point to a specific BMP that implements the Hh regulation of ISC compartment. Studies in mouse developing intestine have shown that Hh can drive the expression of *Bmp4* in mesenchyme [206, 207]. Further, stimulation of E18.5 isolated mesenchyme from small intestine, with increasing dosage of Hh recombinant protein can upregulate BMP4 in a Hh dose dependent manner, while BMP2 levels do not change [141]. *Bmp4* is also abundantly expressed in the intervillus region [180]. Finally, BMP4 and BMP2 can bind the BMPRII receptor (while other BMPs cannot) and mutations of this receptor cause hyperproliferation of the intestinal tissue, increased number of crypts, and development of intestinal polyposis [181] similar to overexpression of the BMP inhibitor Noggin [141, 180]. These observations suggest that in the small intestine, Hh - BMP4-BMPRII regulatory axes maintain the epithelium mesenchyme homeostasis in mouse. Its main function is to restrain the Wnt responding compartment to the base of the crypts and maintain the integrity of the ISC niche.

Several groups provided evidence that also the YAP-induced proliferative response is mediated, at least in part, via activation of Notch and/or Wnt signaling. Downregulation of Hippo-activity resulting in overactive YAP, increased expression of the Notch intracellular domain (NICD) and the target-gene *Hes1* [196, 199]. Moreover, treatment with γ -secretase inhibitors attenuated the phenotype of overactive YAP1, indicating that at least part of the proliferative phenotype is mediated via Notch [196]. Increased Wnt-signaling was demonstrated in *Mst1/Mst2* combined knockouts indicated by higher expression of the Wnt-targets *Jagged1*, *Lgr5* and *Ascl2* [199]. The latter two generally are accepted as intestinal stem-cell markers and *Jagged1* is one of the ligands of the Notch-pathway. The authors suggest that activation of Notch is the consequence of YAP-stimulated β -catenin signaling through expression of *Jagged1* [199].

8 Conclusions

- **Wnt:** The canonical Wnt pathway is the main driving force for the maintenance of the self-renewing phenotype of the intestinal stem cells. Wnt signaling also induces the differentiation of Paneth cells and regulates proper cell migration along the crypt-villus axis via the cell-contact dependent Eph/Ephrin signaling. Decreased expression of the main transcription factor TCF4 has been described in Crohn's disease.
- **Notch:** The cell-contact dependent Notch pathway cooperates with Wnt to preserve the self-renewal of the stem cells. Notch is also involved in the differentiation of absorptive enterocytes vs. the secretory lineage through the lateral inhibition process. Downregulation of the bHLH transcription factor *Atoh1* by *Hes1* is the central event in the inhibition of the secretory phenotype. Increased Notch signaling has been described in IBD.
- **HH:** HH is a critical sensor of epithelial integrity and acts as a switch to proliferation of the epithelium. It is required for stromal cells maturation and localization. It finally promotes maturation of tolerogenic immune cells in the small

intestine. Its activity on stem cell compartment is believed to be indirect as downregulation of Hh causes decrease of BMPs expression and mislocalization of the stromal cells that could be part of the stem cell niche.

- **BMP:** BMP signaling is critical in adult intestinal homeostasis by restraining overproliferation of the intestinal stem cell compartment, acting primarily on the underlining mesenchyme. The direct effect of BMP on ISCs is mediated by PTEN inhibition of AKT, which, in turn, inhibits activation of β -catenin. BMPs support the differentiation of enteroendocrine cells and the full maturation of the secretory cell lineage.
- **Hippo/YAP:** The Hippo pathway is active in post-mitotic differentiated cells leading to nuclear exclusion of YAP, suppressing proliferation. Animal models suggest that Hippo signaling decreases during the regeneration process after experimental colitis.

9 Future perspectives

A number of recent discoveries have provided valuable insight into the mechanisms that regulate the ISC compartment in mouse models, yet there is still a need to perform comparative studies that would define similarities and differences across species, especially with humans. These studies would be mandatory for translational applications. Currently, translation to human physiology and pathology is hampered by the absence of suitable antibodies for many of the currently established ISC markers.

Thus far, the majority of the research on ISC biology in humans has been driven by the observation that mutations in the pathways involved in ISC maintenance occur in the majority of colon cancers. Recent studies reviewed here, however, reported alterations of stem cell pathways components in several non-neoplastic inflammatory driven GI diseases (Table 1). Although very promising, additional studies are required to corroborate these preliminary findings. In particular it will be very valuable to dissect the cause-effect relationship between pathway alterations and pathogenesis. Finally, it will be important to understand how pro-inflammatory stimuli integrate into the network of the signaling governing the GI homeostasis described here. These studies, if pursued, could eventually lead to novel therapeutic approaches to treat inflammatory GI pathologies such as IBD and celiac disease, and could reveal the molecular mechanisms leading to the pathogenesis of dysplasia and cancer in diseases characterized by chronic inflammation.

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Abbreviations

GI	Gastrointestinal
BrdU	Bromodeoxyuridine
LRC	Label-retaining cells
CBC	Crypt Base Columnar cells
ISC	Intestinal Stem Cell
DSS	Dextran Sodium Sulphate
KO	Knockout

FAP	Familial Adenomatous Polyposis
bHLH	basic Helix-Loop-Helix
IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
UC	Ulcerative Colitis
SNP	Single Nucleotide Polymorphism
GSI	γ -secretase inhibitor
SMC	Smooth muscle cells
ISEMF	Intestinal subepithelial myofibroblasts

Highlights

The two stem cell populations in the small intestine have distinct markers.

Critical pathways in the intestine are Wnt, Notch, Hedgehog, BMP, and Hippo/Yap

Alterations of stem cell signaling pathways occur in gut inflammatory pathologies.

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Figure 1.
Schematic representation of the crypt villus axis and the major signaling pathways involved in epithelium homeostasis.

Figure 2.

Network of interactions among the Wnt, Notch, Hh, Bmp4, and Hippo pathways and with other cell signaling components. Dotted lines represent interactions observed in colon cancer cell lines and likely involved in normal stem cell signaling. Solid lines represent interactions proved known to occur in normal ISCs.

Table 1

Alterations in stem cell controlling pathways in GI diseases.

Pathway	Gene	GI Disease	Observations	Ref.
Wnt	<i>TCF4</i>	Ileal CD	Decreased expression, correlating with reduced α -defensin production.	70
	<i>TCF4</i>	Ileal CD	Polymorphism in a putative regulatory region of the promoter.	72
Notch	<i>NOTCH1, HES1</i>	UC	Increased expression.	99, 118, 120
	<i>NOTCH1, DLL4, HES1</i>	CD	Increased expression.	119
	<i>NOTCH1, KLF4</i>	Celiac Disease	Decreased protein levels and consequently goblet cell reduction.	122
Hh	<i>GLII</i>	UC	Polymorphism with decreased functionality of the protein.	144