Hedgehog signaling in homeostasis of the gastrointestinal tract
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Citation for published version (APA):
van Dop, W. A. (2011). Hedgehog signaling in homeostasis of the gastrointestinal tract

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Chapter 1

General introduction

Parts of this introduction have been published in:
**The Hedgehog pathway**

The mechanisms that orchestrate the development of a multi-cellular organism from a single common ancestor cell have fascinated people for centuries. We are still a long way from truly understanding the robust programs that allow building of or our specialized organs that regulate digestion, respiration and hemodynamics and try to guard us from invasion by other unsolicited organisms. However, in the past three decades, many of the genes that play a role in patterning our bodies have been identified and we have learned much about the mechanisms involved in development by characterizing the nature of these patterning genes and the way they work together. One of the pathways that have been shown to play a major role in tissue patterning is the Hh pathway. The embryonically lethal phenotype of Hh mutant mice and the off target effects of the Hh inhibitor cyclopamine have long hampered studies on the role of post natal Hh signaling. The use of conditional and inducible knock-out mice have made it possible to study the role of Hh signaling in adult tissues. In this chapter I will introduce the Hedgehog (Hh) pathway, as well as other morphogen pathways that play a role in maintaining homeostasis in the adult GI tract. In the subsequent chapters I will describe the results of conditionally knocking out components of the Hh pathway, leading to reduced or constitutively active Hh signaling, and the effect this has on the epithelium of the GI tract.

**Morphogens and tissue patterning**

We are built from hundreds of specialized cell types that make up our organs. The development and postnatal maintenance of these cell lineages is tightly controlled and incredibly stable in time. Cells are capable to go through major shifts in protein expression in time in response to physiological and environmental cues without changing cellular phenotype. Each cell type is defined by the expression of a unique combination of proteins transcribed and post-transcriptionally modified from the templates of our 20,000-25,000 genes. One of the central questions in biology is how the information contained in these genes can translate into a spatially complex multi-cellular organism. The idea that such complexity is possible because cellular phenotype is coupled to cellular position is at least 100 years old.1 Most of the evolutionary conserved signaling pathways that act to couple cellular position to cellular phenotype have been first identified in genetic screens in Drosophila most notably the systematic screens performed by Nusslein-Volhard and Wieschaus.2 The type of molecules that have emerged from these genetic screens can be classified in two broad categories, intrinsic and extrinsic factors. Intrinsic signals such as kinases, phosphatases and transcription factors function within the cell and thus act in a cell-autonomous manner. Extrinsic signals are soluble or transmembrane factors that act among cells and regulate cell-nonautonomous processes.3, 4 Extrinsic information is critical for a complex tissue composed of multiple different cell types to organize appropriately. These tissues contain gradients of soluble factors that act in a concentration-dependent manner to control cell fate. Such factors have been termed morphogens. This concept was most clearly voiced by Wolpert in the 1960s,1 who
developed the French Flag model (Figure 1). A morphogen receiving cell has one or more concentration thresholds that result in the expression of a distinct set of target genes. Thus morphogen concentration gradients confer positional information by specifying distinct cellular phenotypes in a field of receiving cells depending on their distance from the source of the signal. Signaling molecules from the Hh pathway, the Wnt-pathway, the transforming growth factor β (TGF β )-superfamily and receptor tyrosine kinase pathways have been shown to act as morphogens in several models of development and are used in the regulation of cell fate throughout evolution and in most developing tissues.

**Hh genes**

The Hh gene was identified in the first systematic screen for patterning genes in *Drosophila*. In larvae of wild type *Drosophila*, a band of bristles called denticles is present across the anterior half of each segment, whereas the posterior half is smooth (the so-called naked cuticle). In screening for mutations that affect the segmental pattern of the *Drosophila* larva, Nüsslein-Volhard and Wieschaus, identified a group of mutants that affected the patterning within the segments but at the same time left the number of segments unaltered. In one of these segment polarity mutants, the posterior half of each segment failed to develop, resulting in a larva that is entirely covered by denticles (Figure 2). This phenotype gave the larva the aspect of a Hedgehog, hence the name. After much effort, the nucleotide sequence of the *Hh* gene was finally obtained and the mammalian *Hh* homologues were identified. Echelard *et al.* identified three *Hh* genes in the mouse. They were named *Sonic Hedgehog* (*Shh*), after a popular Sega computer game character, *Desert Hedgehog* (*Dhh*), after an Egyptian species of Hedgehog, and *Indian Hedgehog*...
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(Ihh), a Hedgehog species endemic in Pakistan. Shh, Dhh and Ihh are highly conserved between mouse and humans. Unfortunately, little is known about factors that control the transcription of Hh genes, particularly in the gut.

Post-transcriptional fate

In order for the Hh protein to act as a potent morphogen, it needs to be processed in different ways. After removal of the signal sequence the approximately 45 kD large Hh proteins are cleaved auto-catalytically yielding a 19 kD N-terminal fragment that contains all the signaling functions, and a 26 kD C-terminal fragment that has no other function than to catalyze the cleavage (Figure 3A). In the course of the cleavage, the SHH protein is covalently palmitoylated and a covalent cholesterol moiety is attached to the C-terminal part of Hh-N. This is essential to both activity of the Hh protein and the signaling range. Despite the fact that lipid modified Hh is not soluble, it is known to form a concentration gradient through a tissue and is able to activate long-range target gene expression over at least 8-10 cell diameters. Different models have been proposed to explain how the lipophilic Hh can spread through an aqueous tissue (Figure 3B). Several laboratories have shown that both cholesterol and palmitate modification are required to mediate multimerization complexes of NH2-terminal Hh proteins. The lipid moieties are thought to be embedded in the core of these complexes, in analogy to micelles. A second model proposes that the lipid moieties of Hh-Np associate with lipoprotein particles.

The receptor complex

Hh signal transduction is highly unusual, containing many features unique to this signaling system. The Hh binding receptor Patched (Ptch) is a 12-transmembrane protein with two large hydrophilic extracellular loops that mediate Hh binding. However, Ptch does not convey the Hh signal to the intracellular components of the
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**Figure 3** SHH protein processing and transportation

(A) Post-translational processing of the full-length Hh protein results in a 19 kD biologically active N-terminal fragment that is lipid modified by cholesterol and palmitate, a reaction that is catalyzed by Skinny Hedgehog. (B) The hydrophobic Hh-Np can form multimers through interactions of the lipid moieties with each other or can bind to lipoprotein particles through the insertion of the lipid moieties into the outer phospholipid monolayer in order to enhance their ability to spread through a tissue.

**Figure 4** The Hedgehog receptor complex

A model of PTCH-SMO interaction. (A) In the absence of Hedgehog PTCH functions as a transmembrane pump that pumps out a SMO inhibitory compound that is yet to be identified. (B) Binding of Hedgehog to PTCH shuts down its pump function and as a result the inhibition of SMO is released.
pathway itself like a conventional receptor. Rather, binding of Hh to Ptch alleviates the inhibitory effect of Ptch on another membrane receptor, the 7-transmembrane protein Smoothened (Smo). Two Ptch genes exist in vertebrates, Ptch1\(^{21}\) and Ptch2,\(^{22}\) but Ptch1 mediates most, if not all, effects of Hh signaling.\(^{23, 24}\) In the absence of Hh, Ptch inhibits signaling by Smo. Upon binding of Hh to Ptch this inhibition is alleviated and Smo will activate Hh target gene transcription (Figure 4).\(^{25}\)

### Signal transduction and target genes

The regulation of Smo activation and downstream Hh signaling has been only partly revealed. In vertebrates the Hh pathway acts through three transcription factors, termed Gli1, Gli2 and Gli3.\(^{26-29}\) Gli2 acts mainly as a transcriptional activator whereas Gli3 can also be processed into a transcriptional repressor.\(^{30-32}\) Gli1 is a transcription factor as well as a target of Hh signaling and functions as an amplifier of activated Hh signaling.\(^{33}\) Primary cilia have been shown to play a major role in Hh signaling in vertebrate systems (Figure 5). When the Hh signaling pathway is activated, all key components of the Hh pathway accumulate in the cilia. In the absence of Hh ligand, Ptch1 is localized at the cell surface of the ciliary base where it waits to bind extracellular Hh ligands. Although Smo circulates in and out of the cillum,\(^{34}\) Smo is mainly localized outside of the cillum at the cell membrane and intracellular vesicles,\(^{35-38}\) Upon binding of Hh to Ptch1, the

![Figure 5 A model of Hh signaling in vertebrates](image)

(A) In the absence of Hh ligand Gli2 and Gli3 are present in small amounts in the cillum and are processed into Gli repressors. (B) Upon stimulation of a cell with Hh ligand Gli2, Gli3 and Smo accumulate at the primary cillum and the Gli proteins become transcriptional activators of the Hh signaling pathway.
receptor-ligand complex is internalized in endosomal vesicles, Smo accumulates at the ciliary membrane and Hh target genes are activated by Gli proteins. Gli2 and Gli3 are the major Glis to transduce the Hh signal in the gut. Gli2 shifts from a primarily cytoplasmic localization to the distal cillum tip and the nucleus, where it activates gene expression. In the absence of Hh Gli3 is restrained to the cytoplasm by Suppressor of fused (Sufu) and proteolysed into a truncated transcriptional repressor. Gli2 can also function as a repressor of Hh target expression, however processing of Gli3 is much more efficient than that of Gli2. Sufu-Gli3 interaction is therefore a major control point in the Hh pathway. Regulation of target genes differs in time and between organs. Expression of Ptch1, Gli1 and Hhip are most widely used as readouts for Hh pathway activity, but many more Hh targets have been described. Ptch1 and Hhip are not only targets of Hh signaling but also part of a negative feedback loop and diminish the Hh response. On the other hand Gli1 functions as transcriptional target which further enhances signaling and thus is part of a positive feedback loop.

Other morphogen pathways in the GI tract

Besides the Hh pathway several other morphogen pathways are important for homeostasis of the GI epithelium. As a possible interaction is described in this thesis between the Hh pathway and the Wnt, TGFβ, Activin and Bone morphogenetic protein (Bmp) pathway, each of them will be briefly introduced below.

Wnt

Wnt signaling is important for the maintenance of the stem cell compartment, which is located at the bottom of the crypts. In mice that lack Wnt signaling, the proliferative precursor cell compartment disappears. Conversely, over activation of Wnt signaling results in hyperproliferation of intestinal crypts. In the intestine different Wnt ligands are expressed, however despite many efforts the location of the source of the main Wnt ligands that mediate effects on proliferation and self renewal remains unknown. Wnt signaling is mediated by β-catenin and nuclear accumulation of β-catenin is often used as a read-out for active Wnt signaling. In the absence of Wnt ligand β-catenin is degraded by a destruction complex that consists of adenomatous polyposis coli (APC), casein kinase I (CKI), glycogen synthase kinase 3 (GSK3), and axin. This complex targets β-catenin for proteasomal degradation through phosphorylation. When Wnt ligand binds to their Frizzled and low-density lipoprotein receptor–related protein (LRP) receptors the destruction complex falls apart and β-catenin is no longer degraded. The β-catenin level in the cell rises, resulting in the translocation of β-catenin into the nucleus. In the nucleus β-catenin replaces Groucho on T cell factor (TCF) transcription factors. β-Catenin/TCF form an active transcriptional complex, leading to the expression of Wnt target genes (Figure 6). Wnt target genes used in this thesis are EphB2, EphB3, Cd44, Olfm4 and Lgr5.
The TGFβ signaling pathway, the Activin pathway and the Bmp pathway are all part of the TGFβ super family and play an important role in development and tissue homeostasis. They regulate growth, apoptosis and differentiation of intestinal epithelial cells and therefore mutations in these pathways are common events in gastrointestinal cancers.49 The signaling pathways are highly conserved and involve 2 receptors (type I and type II) and the signal transducers Smads (Figure 7). There are 8 Smad proteins. Smads 2 and 3 mediate TGFβ signaling and Activin signaling and Smads 1, 5 and 8 mediate Bmp signaling. Together these Smads are called the receptor-Smads. Smad 4 is the co-mediator Smad, which acts as a co-factor for all Smad mediated signaling. Smad 6 and 7 form the inhibitory Smads and negatively regulate signaling by competing with the receptor-Smads for receptor or co-Smad interaction and by targeting the receptors for degradation.50

TGFβ1, TGFβ2 and TGFβ3 are the primary ligands of the TGFβ pathway. Signaling is initiated by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I kinase
domain, which then propagates the signal through phosphorylation of Smad 2 and 3. These form a complex with Smad 4 upon which the activated Smad complexes are translocated into the nucleus where it regulates transcription of specific TGFβ, Activin or Bmp target genes.

Activin signaling is very similar. The ligands for Activin signaling are dimeric proteins and are composed of two Inhibinβ subunits. Inhibins, antagonists of Activins, are composed of an Inhibinα and an Inhibinβ subunit. There is 1 α subunit and there are four different genes known to encode for the β subunits: Inhibinβα, Inhibinβb, Inhibinβc and Inhibinβe. Activins bind to type II Activin receptor leading to the recruitment, phosphorylation and activation of type I Activin receptor. The activated type I receptor phosphorylates Smad 2 and Smad 3. This means that Smad 2 and Smad 3 are signal transducers for both the TGFβ pathway and the Activin signaling pathway. The phosphorylated Smad2 and Smad3 form a complex with Co-Smad (Smad4), and the resulting Smad complex accumulates in the nucleus, binds to the promoter region of the
target genes, and regulates their expression. Goosecoid is a conserved target of Activin signaling\textsuperscript{52} and is used in this thesis as a readout for Activin pathway activity.\textsuperscript{53} Up till now little is known about the potential role of Activins in the adult intestine.

There are many different Bmp ligands, but Bmp 2, 4 and 7 are the major Bmps produced in the intestine.\textsuperscript{54-57} A link between Bmp signaling and Wnt signaling has been previously described.\textsuperscript{58, 59} BMPs and their receptors are expressed in both epithelial and mesenchymal compartments and both epithelial and mesenchymal Bmp signaling are known to contribute to the maintenance of the intestinal stem cell niche.\textsuperscript{54, 55, 58-62} Bmps initiate signaling by binding cooperatively to receptors type I and II triggering the phosphorylation and activation of the type I receptor by the type II receptor kinase.\textsuperscript{50} Smads 1, 5 and 8 are then phosphorylated and form a complex with Smad 4. This complex translocates into the nucleus where Bmp target genes are expressed of which Id 1-4 are examined in this thesis.

**Hh signaling in development of the GI-tract**

The gastrointestinal tract develops from a simple tubular structure composed of cells derived from all three germ layers. Cells from the endoderm will form the epithelial layer; mesodermal cells differentiate into mesenchymal cell types such as myocytes and fibroblasts whereas neural cells originate from the ectoderm and innervate the gastrointestinal tract. The cells that form the gut tube acquire regional specialization along different axes of the developing embryo. The longitudinal axis restricts the fate of cells that form the gut tube to become part of the foregut (esophagus, trachea, lungs, thymus, stomach, liver and pancreas), the midgut (small intestine) or the hindgut (colon and anus). A second axis of specialization is the radial or vertical axis, along which the constant renewal of epithelial cells in the adult tissue takes place. Most patterning mechanisms along these axes involve cross-talk of morphogens between cells of the different germ layers. Hh signaling plays a role in the specification of cellular fate along both the longitudinal and the vertical axes. The role of Hh signaling and its interaction with other morphogen pathways in patterning of the developing embryo have taught us much about its role in adult tissue homeostasis. As the subsequent chapters focus on adult tissue homeostasis, this introduction will be confined to Hh in the adult GI tract. For a comprehensive review on the role of Hh signaling in development of the GI tract, see ref. 39\textsuperscript{39}. 
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Hh signaling in homeostasis of the adult GI tract

*Morphostasis in general*

Although adult tissues seem to be static and stable, many tissues are not. They have a very high cellular turnover rate and their micro-architecture can be disturbed for example in chronic inflammation. The GI tract is a good example of the importance of positional information in cellular renewal in the adult. At each level of the gut a common precursor cell produces a module of specialized cell types appropriate for its position along the anterior-posterior axis. These modules display elaborate patterns of differentiation along the radial axis. At some parts of the GI tract precursor cells generate two functionally different compartments of cells that migrate in opposite directions (e.g. the stomach). Although stability of the resulting complex epithelial micro-architecture is the norm, instability is frequently observed in humans. This instability leads for example to the formation of metaplasia, atrophy or hyperplasia such as in polyposis. Often these instable epithelial states are at a high risk to progress to gastrointestinal cancer. This may explain why mutations in morphogenetic pathways, such as the Wnt and BMP pathway can initiate carcinogenesis.58, 59, 62, 63 Gastrointestinal epithelial cells undergo a well-controlled maturation process. Precursor cells generate descendants that withdraw from the cell cycle and induce transcription of cell lineage specific proteins. When the cell is fully differentiated it is shed into the gut lumen or undergoes apoptosis to maintain cellular census. The proliferative compartment of precursor cells receives negative feedback information from cells in the differentiated compartment. Through such negative regulation the amount of differentiated cells can regulate the pace of their own replacement. If differentiated cells in the superficial epithelium are lost by damage such a mechanism allows for increased proliferation to guard epithelial integrity. The mediator(s) of this negative feedback loop have not been identified to date but are clearly soluble molecules capable of long range signaling. The identification of the extrinsic controls of cell fate is fundamental to the understanding of the regulation and deregulation of epithelial homeostasis. Hh is one of these extrinsic regulators and its role in homeostasis in the adult GI tract will be described below.

*Esophagus*

Up until now very little attention has been paid to the role of Hh signaling in homeostasis of the adult esophagus. The few studies that describe Hh signaling in the normal esophagus show conflicting results on which cell type expresses Hh and Hh signaling targets and which of the Hh homologue(s) is/are expressed. 64, 65 As will be demonstrated in chapter 4, we found that Shh is the only Hh homologue expressed in the mouse esophagus and that Hh targets are expressed by the basal layer of the esophageal epithelial cells. This topic needs to be further investigated in the future to be able to draw firm conclusions concerning the role of Hh signaling in esophageal homeostasis.
Stomach

Renewal of gastric epithelial cells is a bi-directional process. The gastric mucosa is a flat surface that contains multiple invaginations or gastric units. Recently Barker et al.\textsuperscript{66} identified \textit{Lgr5} positive stem cells at the base of the glands throughout the pyloric region and limited number of glandular units adjacent to the esophagus and squamous forestomach boundaries. No \textit{Lgr5} positive stem cell populations were found in the adult corpus, indicating the existence of other \textit{Lgr5} negative stem cell pools.\textsuperscript{66} Although at least in part of the gastric glands the stem cells may be located at the bottom of the glandular units, the main proliferating precursor cell compartment is located in the isthmus (Figure 8), somewhere approximately halfway up these units.\textsuperscript{67, 68} Cells that migrate towards the luminal surface secrete the protective soluble mucin MUC5AC and form the gastric pit (the upper part of the unit).\textsuperscript{69} Cells that migrate downwards form the gastric glands. The proximal glandular stomach (fundus) has small pit regions and large glands, whereas the distal stomach (antrum) has large pit regions and small glands. Fundic glands are composed of parietal cells which secrete acid, endocrine cells, such as somatostatin producing D-cells and gastrin producing G-cells, and mucous neck cells, a

![Figure 8: Hh signaling in the adult stomach](image)

From the proliferating cell compartment located at the isthmus cells migrate either downwards towards the gland, or upwards towards the foveolar region. Ihh modulates gastric pit cells, Shh drives differentiation of progenitor cells into various cell lineages.
cell type with uncertain function and which trans-differentiates when halfway down the
gland into zymogenic cells that secrete digestive enzymes.\textsuperscript{70} Antral glands are exclusively
composed of mucous and endocrine cell types.\textsuperscript{71} In this homeostatic system factors have
to control asymmetric cellular differentiation where the appropriate amount of cells
adopts either a pit or gland cell fate. The presence of such factors that act as polarizing
signals in the gastric units is clear from histopathological observations in patients
with hypertrophic gastropathies. These gastropathies include Ménétrier disease and
hyperplastic hypersecretory gastropathy (HHG).\textsuperscript{72} In Ménétrier disease Transforming
Growth Factor (TGF)\textsubscript{a}, a ligand for the EGF receptor, is over-expressed leading to excess
proliferation and differentiation of pit cells and loss of glandular epithelial cells.\textsuperscript{73-75} In
contrast, the hypertrophy in patients with HHG is characterized by an increased mass of
gland cells. Shh is broadly expressed by all epithelial cell lineages of the gastric gland.\textsuperscript{76-78}
It is highest in parietal cells very close to the precursor cell compartment and gradually
diminishes to very low levels as parietal cells migrate to the bottom of the fundic gland.\textsuperscript{77}
Several studies have now shown that Shh is necessary for progenitor cell differentiation
into various gastric cell lineages rather than for proliferation.\textsuperscript{79} Ihh appears to modulate
gastric pit cells.\textsuperscript{79, 80} Hh signaling in the adult mouse stomach is exclusively paracrine,
from epithelium to mesenchyme.\textsuperscript{76}

Reduced levels of Shh expression have been observed in gastric atrophy, a condition
often caused by chronic inflammation such as in \textit{H pylori} infection. Atrophic gastritis
is characterized by thinning of the mucosa, progressive loss of glandular cell types,
hyperproliferation of the foveolar compartment and ultimately the development of
metaplasia. Furthermore it is characterized by a pronounced loss of gastric acidity
(hypochlorhydria). Mice in which parietal cells are specifically ablated present with loss
of gastric gland lineages and expansion of the foveolar compartment.\textsuperscript{81} This suggests
that parietal cells secrete a factor that regulates the cell fate of the other gastric cell
lineages. One of the parietal cell-derived factors that may very well play a role in the
development of atrophic gastritis is hydrochloric acid. Loss of gastric acidity results in a
similar phenotype as depletion of parietal cells. Another candidate is Shh, which is also
expressed by parietal cells.\textsuperscript{82} Loss of Shh is correlated with the degree of atrophic gastritis
and intestinal metaplasia in \textit{H pylori}-infected patients and Mongolian gerbils.\textsuperscript{83, 84} Zavros
et al. have shown that gastric Shh expression depends on circulating gastrin serum levels
and gastric acidity.\textsuperscript{85} This suggests Shh expression may act in parallel with, or up- or
downstream of hydrochloric acid. Two groups have further investigated the role of Shh
in the stomach. Waghray et al.\textsuperscript{78} have shown that Shh expression is progressively lost
upon infection with \textit{H. felis} in mice. More importantly, they found that this loss was
not merely a result of loss of gland cell types, but actually preceded it. They treated
their mice with omeprazole, a proton pump inhibitor, and Interleukin-1\textbeta (Il-1\textbeta), one
of the key cytokines in \textit{H pylori} associated gastric cancer development.\textsuperscript{86} and a potent
inhibitor of gastric acid secretion.\textsuperscript{87, 88} Both Il-1\textbeta and omeprazole lead to a reduction in
Shh expression, however, suppression of Shh expression by Il-1\textbeta and omeprazole was
additive, suggesting that they are mediated at least in part by parallel pathways.\textsuperscript{78, 89} Xiao et al. examined the role of Hh signaling in the stomach through generation of a parietal cell-specific knock-out for Shh (\textit{HKCre-ShhKO}). These mice presented with similar phenotypic changes, such as foveolar hyperplasia and glandular atrophy, which were also previously found in both parietal cell depleted mice and hypergastrinemic mice.\textsuperscript{81, 82} They demonstrated that histamine-stimulated acid secretion is severely compromised and hypergastrinemia develops. Octreotide, a somatostatin analog that inhibits the secretion of gastrin, entirely reversed the molecular and morphological changes in the \textit{HKCre-ShhKO}. This suggested that effects of loss of Shh on gastric epithelial morphology were to a large degree indirect and the result of hypergastrinemia. In conclusion, parietal cells secrete factors that affect differentiation of the other gastric cell lineages and are lost in atrophic gastritis. Both hydrochloric acid and Shh seem to be such factors and their regulation seems to be intimately linked.\textsuperscript{78, 89, 90}

\textbf{Intestine}

The small intestinal epithelium is a homeostatic self-renewing tissue, ordered into crypts and villi. In the mouse, crypt formation is completed in the third post-natal week. Intestinal stem cells are located at the bottom of the crypts. They give rise to transit amplifying cells which proliferate rapidly and differentiate into the four cell lineages of the small intestine. Enterocytes, goblet cells and entero-endocrine cells migrate up from the precursor cell compartment towards the villus whereas Paneth cells stay at the base of the crypts (Figure 9). The organization of the colon is somewhat similar to that of the intestine, with the difference that it lacks villi and Paneth cells. Barker et al. showed that Lgr5 is a marker of intestinal stem cells and that it is expressed by the crypt based columnar cells.\textsuperscript{47} The precursor cells and the differentiated cells are in a dynamic equilibrium in order to balance the rate of proliferation with cell loss at the epithelial surface. This balance depends on the presence of negative feedback loops and Hh signaling has been shown to play a major role in this loop. During development both Shh and Ihh are expressed in the intestinal epithelium. Conflicting data exist on the expression of Shh in the adult intestine. Shh in the adult mouse intestine was not detected by in situ hybridization,\textsuperscript{91} but it has been shown using a \textit{ShhGfp} reporter mouse that low levels of Shh may be expressed by rare cells at the crypt base.\textsuperscript{92} Although the role of Shh is not fully clear yet, it has been shown by several groups that Ihh is the major Hh expressed in the intestine and is secreted by the differentiated enterocytes.\textsuperscript{93-95} It has been shown that inhibition of Hh signaling with either cyclopamine,\textsuperscript{94} or by genetic disruption, results in up-regulation of Wnt signaling, increased proliferation of precursor cells and disturbed maturation of enterocytes.\textsuperscript{95-97} On the other hand, conditional activation of Hh signaling reduces Wnt signaling and proliferation of precursor cells and results in premature commitment of these cells to the enterocyte lineage (Figure 9).\textsuperscript{91} Hh signaling in the intestine is exclusively paracrine,\textsuperscript{96, 91} from epithelium to mesenchyme. Hh-responsive cells in the adult intestine include smooth muscle precursor cells, smooth muscle cells, myofibroblasts and pericytes.\textsuperscript{76} Several studies have demonstrated that
in the adult intestine increased Hh signaling leads to accumulation of smooth muscle cells\textsuperscript{76, 91} and reduced Hh signaling results in depletion of smooth muscle cells from the villus core.\textsuperscript{95, 97} This indicates that in the adult intestine Hh signaling controls the size of the smooth muscle cell population in the villus core, as has also been shown in the developing intestine.\textsuperscript{98} However, it has not been resolved yet which factors are expressed, or suppressed, by these Hh-target cells in order to inhibit Wnt signaling. Hh signaling regulates Bmp signaling in the developing and adult intestine,\textsuperscript{91, 94, 95, 99} and a link between Bmp and Wnt signaling has been previously described. For example, in \textit{Bmp-receptor1a} conditional mutant mice\textsuperscript{58} and in transgenic mice that express the Bmp antagonist Noggin under control of the Villin promoter\textsuperscript{59} reduced Bmp signaling lead to enhanced Wnt signaling and accumulation of precursor cells. However, since the Bmp pathway is not normally active at the base of the crypts, it is unlikely that Bmps are the main negative regulators of Wnt signaling. In this thesis we propose a potential role for other mesenchymal factors, such as Activins, family members of the TGF\textit{b} pathway. Activin signaling is mediated through pSmad2,3, which are active in normal crypts, but lost in \textit{Ihh} mutant mice.\textsuperscript{95} Loss of pSmad2,3 was associated with a
down-regulation of Inhibinβs, which encode for the subunits that form the Activins. Therefore, loss of Activin signaling may be the cause of increased Wnt signaling and epithelial proliferation in mice with reduced Hh signaling, but this possibility still has to be further investigated.

**The link between Hh signaling and inflammatory bowel disease**

Genetic variants in the Hh pathway have been linked to the development of inflammatory bowel disease. Lees et al.\textsuperscript{100} identified a germ-line variant in \textit{GLI1}, encoding a GLI1 protein with reduced function, that was associated with the development of IBD. In addition they found that Hh pathway components were down-regulated in colonic inflammation and that mice with reduced Gli1 activity were more susceptible to DSS induced colitis. The authors found that myeloid cells, including dendritic cells, are direct targets of Hh signaling during both homeostasis and under inflammatory conditions.\textsuperscript{100} The role of Hh as an anti-inflammatory signal in the intestine, has also been shown in different conditional mouse models.\textsuperscript{95-97} In the bi-transgenic \textit{VFHhip} mice (\textit{12.4KVillinCre x 12.4KVillinLacZfl/flHhip}) the pan-Hh inhibitor \textit{Hhip} is expressed perinatally and by 1 month of age Gli1 expression is reduced to approximately 30% of normal expression.\textsuperscript{97} In the mouse model we examined and described in this thesis, loss of Ihh expression could be induced specifically in the adult small intestinal epithelium by injecting \textit{Cyp1a1Cre-Ihhfl/fl} mice with naphtoflavone. The models showed expansion of the proliferative crypt region with lengthening of the crypts, loss of smooth muscle cells from the villus core and eventually villous atrophy and inflammation in the lamina propria. Zacharias et al. cultured small intestinal mesenchyme without the presence of the epithelium, the source of Hh, and showed that after 48 hours pro-inflammatory genes are up-regulated. By adding recombinant Hh protein Hh signaling could be reactivated within 24 hours and the levels of pro-inflammatory cytokines were reduced, suggesting Hh is an important anti-inflammatory signal in the intestine. In our own experiments in the \textit{Cyp1a1Cre-Ihhfl/fl} mice\textsuperscript{95} we found that in addition to the epithelial characteristics of regeneration such as crypt lengthening and fissioning loss of Ihh resulted in influx of fibroblasts and macrophages into the villus mesenchyme, another hallmark of a wound healing response. After prolonged loss of Ihh expression progressive inflammation was observed with influx of macrophages, T-cells and neutrophils into the inter-crypt spaces and the development of extensive intestinal fibrosis. Thus loss of Ihh expression which would normally result from damage to the intestinal surface epithelium seems to initiate an intestinal wound healing response which if unresolved (such as in the Ihh mutant mice in which Ihh is permanently lost) leads to the development of chronic inflammation and fibrosis. In both models the observed villous atrophy strongly resembles histology observed in patients with celiac disease. However no increase in intra-epithelial lymphocytes was observed, whereas accumulation of these cells is a hallmark of celiac disease. This makes loss of Ihh as a causal role for the development of celiac disease unlikely.
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In conclusion, Hh is not only a negative regulator of intestinal precursor cells, but also an anti-inflammatory modulator of the mesenchymal milieu. Unresolved loss of Ihh from the intestinal epithelium ultimately leads to loss of smooth muscle cells from the villi, mucosal damage, villous atrophy and chronic enteritis with intestinal fibrosis. Ihh can therefore be seen as an important indicator of epithelial integrity.

Hh signaling and cancer

It is becoming more and more evident that disturbance of cellular interpretation of extrinsic information is a major step in the initiation of GI malignancies. Several morphogenetic pathways have been implicated for example in the development of colorectal cancer. First, mutations in the WNT pathway were identified as the cause of the Familial Adenomatous Polyposis (FAP) syndrome and most sporadic colonic adenomas. Then hamartomatous polyposis syndromes such as Juvenile Polyposis were linked to mutations in the BMP receptor and the related intracellular signal transducer SMAD4. The role of Hh signaling in carcinogenesis has become evident from inherited cancer syndromes and genetically modified mice. Mutations in the Hh receptor Ptc1 lead to constitutive activity of the Hh pathway and cause basal cell carcinomas (BCC) and medulloblastomas. However, in Hh pathway mutant mice analyzed thus far no gastrointestinal cancers have developed that resemble human cancers. This indicates that in the gastrointestinal tract, over activation of Hh signaling by itself is insufficient to initiate carcinogenesis and mutation of additional genes, such as the oncogene Kras, is necessary for cancer development. Although Hh is often called an oncogene, in the stomach Shh is necessary for cell differentiation rather than for proliferation and this thesis demonstrates that Ihh is an anti-proliferative signal throughout the whole intestine and thus in this case is likely to function as a tumor suppressor gene rather than an oncogene.

Hh signaling is increased in gastric tumors and possibly also in adenomas and adenocarcinomas of the human colon and Hh activation in gastric tumors correlates with advanced stage and poor differentiation. This suggests that, although Hh signaling has no role in initiation of cancer development in the GI tract, it can play a role in cancer progression. Apparently in the GI tract the role of Hh signaling in homeostasis of healthy tissue is different from its role in carcinogenic tissue. This difference could be derived from differences in pathway utilization between the normal and tumorigenic contexts, but this will have to be further investigated in the future.
Concluding remarks and outline of the thesis

The use of inducible and conditional mutant mice and reporter mice has greatly improved analysis of the role of Hh signaling in the adult gastrointestinal tract. It has become clear that in normal homeostasis of the stomach and throughout the whole intestine Hh signaling is exclusively paracrine. In chapter 2 and 3 a clear role for Hh signaling in homeostasis of the adult intestinal epithelium is described. The use of inducible knock-out mice has now proven that in the mouse Hh signals via the mesenchyme to the epithelial cells at the bottom of the crypts where it negatively regulates Wnt signaling and proliferation of precursor cells and positively regulates enterocyte differentiation. The factor(s) in the mesenchyme that mediate these effects are still unclear and identifying these will be topic of further research. Furthermore, besides a role in epithelial homeostasis it seems that Hh signaling also has a role in immunological homeostasis of the intestine. Genetic variants in this pathway have been linked to the development of IBD and we demonstrate in chapter 3 that loss of Hh signaling in mice causes spontaneous development of chronic enteritis. This link with inflammation is an interesting new aspect of Hh signaling in the adult gastrointestinal tract. Despite the growing interest in Hh signaling in the adult stomach and intestine, the role of Hh signaling in the adult esophagus has deserved relatively little attention up till now. We describe our first results on this topic in chapter 4. Chapter 5 is a side step towards the immunology and describes that by preventing the recruitment of leukocytes in a mouse model for colitis the degree of colitis can be reduced.

I hope this thesis will convince the reader that morphogens are not only important in development of the GI tract, but are essential throughout life in maintaining tissue homeostasis. Their link with inflammation and progression of cancer in the GI tract makes them interesting topics for further investigation, both in the lab and in the clinic.
Chapter 1

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