Hedgehog signaling in homeostasis of the gastrointestinal tract
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**Chapter 1**

**Figure 8  Hh signaling in the adult stomach**
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.

**Figure 9  Hh signaling in the adult intestine**
In the adult intestine transit amplifying cells either move downward to form Paneth cells (only in the small intestine), or upward to form the other intestinal cell lineages. Ihh is the predominant Hh expressed in the adult intestine and signals to the mesenchyme.
Figure 1. Localization of Hh pathway components in the adult mouse colon by in situ hybridization and immunohistochemistry. Ihh (A, left) mRNA was expressed in the epithelial cells of the midcrypt region. Shh (A, right) mRNA was not detectable in the colon, while the neural tube of an 11 day old embryo showed the expected ventral staining (inset in A, right). Ihh protein was expressed by the differentiated cells at the top of the crypts (B). Expression of Ptch1 (C) and Gli1 (D) mRNA was restricted to the mesenchyme underlying the epithelium at the upper part of the crypts in control mice. Many of the Ptch1 positive cells had the typical characteristics of subepithelial myofibroblasts (arrows in C).

Figure 2. Successful recombination of Ptch1 and activation of Hh signaling in conditional mutant mice. An in situ probe directed against exon 8 and 9 of Ptch1 mRNA showed successful deletion of these two exons in the Ptch1 mutant animals (A). Recombination efficiency at the Ptch1 locus in the colon was 99% ± 0.3% (B). A probe against exons 1-6 of Ptch1 mRNA (not excised upon activation of the Cre enzyme) showed that the Ptch1 mutation resulted in upregulation of this Hh target in the mesenchyme (C). This was confirmed by quantitative RT-PCR (D, P<0.001, n=12 control versus 11 mutant mice). Gli1 was uniquely expressed in the mesenchyme at the upper half of the crypts in control mice and was increased in Ptch1 mutant animals (E and F, P<0.0001 n=12 control mice versus 11 mutant mice). Magnification: 160x (A-D).
**Figure 3.** Hypoplastic crypts and premature commitment to the enterocyte lineage in *Ptc1* mutant animals. H&E staining of colon of control mice (A, left) and *Ptc1* mutant animals (A, right) showed large areas of colonic crypt hypoplasia in the mutant mice at 19 days after the first tamoxifen injection. The bases of the crypts were broadened and contained more highly polarized cells. Electron microscopy revealed that the base of the crypts of the control mice (B, left) contained undifferentiated cells with large round dark-stained nuclei and little cytoplasm. In the *Ptc1* mutant mice (B, right) these cells were partly replaced by more differentiated cells with smaller nuclei and more cytoplasm filled with small vacuoles. Magnifications: 160x (A), 800x (B).

**Figure 4.** Premature differentiation of the epithelial precursor cells. Immunohistochemistry for Ca II (A) and Cdx2 (C) showed that the expression of these differentiation markers was increased in the *Ptc1* mutant mice and extended more towards the base of the crypts. This was confirmed by quantitative RT-PCR on colonic homogenates from control (n=12) and mutant (n=11) mice for Ca II (B, \( P=0.047 \)) and Cdx2 (D, \( P=0.02 \)). No difference was observed in expression of Villin protein or mRNA (E,F, \( P=0.27 \)). Magnifications: 100x (A, C, E).
Figure 5. Accumulation of myofibroblasts in Ptch1 mutant animals. Immunohistochemistry for α-Sma (A) showed accumulation of myofibroblasts in the Ptch1 mutant mice. This was confirmed by quantitative RT-PCR on colonic homogenates from control (n=12) and mutant (n=11) mice (B, \( P=0.007 \)).

Figure 6. Depletion of proliferating precursor cells and reduced Wnt signaling in Ptch1 mutant mice. Immunohistochemistry for Ki67 in control (n=14) and Ptch1 mutant (n=11) mice (A) showed loss of Ki67-positive nuclei in the mutant mice. In some crypts the whole precursor cell compartment was depleted of Ki67 positive cells (arrows in A). Ki67 positive cells were counted per crypt in the distal colon (B) and the proximal colon (C) 19 days after the first tamoxifen injection. A significant reduction in proliferating precursor cells was observed in both segments (\( P=0.0002 \) and \( P=0.003 \) respectively). (D) analysis of proliferation at different time points showed that the reduction in proliferation was first apparent at 10 days after recombination. Immunohistochemistry for β-catenin (E) showed staining of membrane-bound β-catenin all throughout the crypt in both control and mutant mice. In the control mice (n=6) β-catenin accumulated in the cytoplasm and nucleus of cells at the base of the crypts (arrows in E), the hallmark of active Wnt signaling. Nuclear staining of β-catenin was strongly reduced (\( P=0.0006 \)) in the mutant mice (n=6) (E and F). Magnifications: 200x and 400x.
Figure 7. Mesenchymal induction of Hh signaling results in a reciprocal increase of epithelial Bmp signaling. Immunohistochemistry for pSmad1,5,8 (A) showed increased phosphorylation of the Bmp specific Smad1,5,8 in the epithelium of Ptch1 mutant mice. Whereas the base of the crypts of control animals showed no or very little Smad1,5,8 phosphorylation (A, left), phosphorylation was increased throughout the epithelium but most notable at the base of the crypts in mutant mice (A, right). A similar pattern was observed for Id2 (B). Quantitative RT-PCR for Id2 and Id4 (C), both targets of Bmp signaling, confirmed the increase in Bmp signaling ($P=0.03$ and $P=0.01$ respectively). Magnifications: 200x and 400x.
Figure 8. In situ hybridization for Bmp2 (A) showed that Bmp2 mRNA was expressed by the differentiated epithelial cells at the top of the crypts. Bmp4 (B) mRNA was expressed in a graded pattern from the base to the top of the crypt and in the mesenchyme. Bmp7 (C) was expressed by the mesenchyme underlying the epithelium at the upper parts of the crypts. Quantitative RT-PCR on colonic homogenates from control (n=12) and mutant (n=11) mice (D) showed an induction of Bmp2 (P=0.01), Bmp4 (P=0.04) and Bmp7 (P=0.03). Proposed model of negative feedback signaling in the intestinal epithelial crypt (E). Ihh secreted by the differentiated cells acts on the myofibroblasts which secrete factors such as Bmps that subsequently negatively regulate precursor cells at the base of the crypt. Magnification: 200x.
Figure 1. Loss of Ihh signaling from the small intestine in β-naphthoflavone injected Cyp1a1-Cre-Ihhfl/fl adult mice. (A) In situ hybridization showed that Ihh mRNA was exclusively expressed by the epithelial cells on the villi. Expression was highest at the crypt villus junction (arrows in A) and diminished towards the villus tip. (B) Immunohistochemistry for Ihh showed expression of Ihh protein by the enterocytes on the villi in mice injected with solvent whereas β-naphthoflavone injected mice have lost Ihh expression at 2 weeks after treatment (B, right panel). Quantitative RT-PCR for Ihh at different time points (C) and Hh signaling targets Gli1, Hhip, Ptch1 and Ptch2 two weeks after recombination (D, black bars) on intestinal homogenates of β-naphthoflavone injected Ihhfl/fl control and Cyp1a1-Cre-Ihhfl/fl mice confirmed loss of Ihh expression. Original magnifications: 100x. (E) X-gal staining of the duodenum (dd) and ileum (il) of Cyp1A1-stopfl/fl/LacZ mice injected with vehicle (control) and β-naphthoflavone (mutant).
Figure 2 Loss of Ihh is sufficient to initiate a regenerative response. (A,B) A strong increase in the rate of crypt fissioning was observed (arrows in A show fissioning crypts), which was maximal two weeks after recombination (B). (C) H&E staining of the duodenum demonstrated increased crypt density, deepening of crypts and lengthening of the villi in the Ihh mutant mice one month after injection with β-naphthoflavone. (D) Measurements of crypt density and crypt length confirmed an increase in the Ihh mutant mice one month and four months after recombination respectively. (E) Immunohistochemistry for BrdU at one month after recombination. (F) BrdU positive cells were counted and set out both as absolute number of positive cells per crypt and as labeling index, an indication for the percentage of cells per crypt that were positive for BrdU. Original magnifications: 100x (A left panel and C) and 200x (A right panel and E).
Figure 3 Increased Wnt signaling upon loss of Ihh. (A) Immunohistochemistry for β-catenin. (B) An increase in the number of positive nuclei per crypt was observed in the Ihh mutant mice. In situ hybridization for Lgr5 (C) and Olfm4 (D) showed an increase in cells positive for these stem cell markers 1 month after recombination. (E) Incomplete formation of microvilli in the mutant mice one month after recombination. (F) Alkaline phosphatase was still present two months after recombination, but had disappeared from the remaining villi four months after recombination. Original Magnifications: 400x.
Loss of Ihh signaling leads to reduced Bmp and Activin signaling in the epithelium of Ihh mutant mice and increases Tgfβ signaling. (A) Immunohistochemistry for Bmp associated pSmad1,5,8 showed nuclear staining in the villi of control mice, whereas the crypts were negative. One month after recombination the staining was reduced in Ihh mutants. (B) Quantitative RT-PCR for Bmp2, Bmp4, Bmp7 and (C) targets Id1-4, demonstrated a significant reduction in the expression of Bmp4 which correlated with reduced Id1 and Id3. (D) Immunohistochemistry for Tgfβ/Activin associated pSmad2,3 showed exclusive signaling by Smad 2 and 3 in the epithelial cells of the crypts. Upon loss of Hh signaling the staining in the crypts was lost. (E) Quantitative RT-PCR demonstrated a non-significant up-regulation of the Tgfbs, mainly of Tgfβ1, and a significant up-regulation of Pai-1, a target of Tgfβ signaling. (F) Quantitative RT-PCR for the different Inhibins demonstrated that Inhibina, which is needed to form Inhibins, was up-regulated, and that the different Inhibins (ba, bb, bc and be, which are subunits needed to form the Activins), were down-regulated. Original magnification: 100x (large panels in A and D) and 400x (enlargements in A and D).
Figure 5 Influx of macrophages and fibroblasts into the villus core. (A) H&E stainings demonstrated increased cellularity of the villus core one month after recombination compared to the control group. An influx of macrophages (F4/80 positive cells, B), vimentin positive cells (C) and fibroblasts (S100A4 positive cells, D) was observed at this time point. Counting of macrophages, vimentin positive cells and fibroblasts showed recruitment from the earliest time point examined (graph in B-D). (E) A double staining for vimentin (green) and Cd68 (red) showed no double staining one month after recombination. (F) A double staining for vimentin (red) and S100A4 (green) demonstrated that part of the vimentin positive cells were also S100A4 positive (white arrows in F). Original Magnifications 400x.
Figure 6 Loss of Ihh resulted in the development of chronic enteritis with villous atrophy. (A) Compared to control duodenum the duodenum of Ihh mutant mice showed the development of a chronic inflammatory infiltrate with partial loss of villi. Crypts displayed increased depth and fissioning (arrows) as was also observed at earlier time points. In the ileum (B) epithelial damage was more severe and loss of villi was almost complete. In some parts of the ileum the epithelium had entirely covered the damaged mucosal layer (arrows), giving the crypts a buried appearance. At 6 months after recombination at some places erosion (C) had developed. The Ihh mutant mice, which where behind in weight gain from the moment of recombination, started losing weight at five months of recombination (D). Original magnifications: 100x (left panels in A-C) and 200x (enlargement in A).
Figure 7 Infiltration of inflammatory cells and development of intestinal fibrosis in the \textit{Ihh} mutant mice. (A) Four months after recombination an inflammatory infiltrate appeared in the crypt area of the \textit{Ihh} mutant mice, consisting of macrophages (F4/80 positive), neutrophils (Ly6G positive) and T-cells (Cd3 positive). Progressive accumulation was observed of collagen (B) and fibronectin (C) in the lamina propria. Original magnifications 400x (A), 200x (B,C).
Supplementary Figure 2. EphB2, EphB3 and Cd44, targets of Wnt signaling were all up regulated in the Ihh mutant mice one month after recombination. Original magnification: 400x.

Supplementary Figure 3. (A) Immunohistochemistry for lysozyme, a Paneth cell marker, one month after recombination. A modest increase in lysozyme positive cells was observed at the base of the crypts of mutant mice one month after recombination. We found 3.2 positive cells per crypt (n=8) versus 4.1 positive cells per crypt in the Ihh mutant mice (n=5, P=0.015). (B) Immunohistochemistry for Alcian Blue, a Goblet cell marker, one month after recombination. There relative increase in Goblet cells was not significant (P=0.06, n=5 for both control and mutant group). (C) Immunohistochemistry for Chromogranin A, a marker for entero-endocrine cells, one month after recombination. No difference was found in relative amount of entero-endocrine cells between the control and the mutant mice (P=0.8, n=5 for both groups). Original magnification: 200x.
Supplementary Figure 4. Immunohistochemistry for Bmp signaling target Pai-1 showed increased levels of Pai-1 in the mesenchyme of mutant mice 1 month after recombination. Original magnification: 400x.

Supplementary Figure 5. An immunofluorescent double staining for \( \alpha \)-Sma and desmin in control mice. The double staining demonstrated that most cells in the villus core were either smooth muscle cells (desmin single positive cells, open arrows) or smooth muscle precursor cells (desmin-\( \alpha \)-Sma double positive cells, closed arrows). The triple arrow indicates an \( \alpha \)-Sma single positive cell. Original magnification: 400x.

Supplementary Figure 6. Loss of smooth muscle cells from the villus core in \( Ihh \) mutant mice. \( \alpha \)-Sma positive cells were reduced in number at one month after recombination and had disappeared at the two and four months time points (A). Desmin positive cells were present in the villi until two months after recombination and disappeared at four months after recombination (B). (C,D) \( \alpha \)-Sma and desmin positive cells had an elongated appearance in the control mice. In the \( Ihh \) mutant mice, 1 month after recombination, the remaining \( \alpha \)-Sma positive cells and the desmin positive cells had lost their elongated structure and seemed to roll up into a sphere like shape. Original magnifications: 200x (A,B) and 400x (C,D).
Supplementary Figure 8 The number of Cd3 positive intraepithelial lymphocytes went down from 21.7 cells in control mice (n=8) to 3.5 cells in mutant mice four months after recombination (n=4, P<0.001) and the number of Cd3 positive cells in the villus core went down from 26.1 positive cells in control mice (n=8) to 9.3 positive cells in the mutant mice four months after recombination (n=4, P<0.001). Original magnifications: 400x (A) and 200x (B).

Chapter 4

Figure 1. Localization of Hh pathway components in the adult mouse esophagus. (A) Quantitative RT-PCR for the different Hh homologues Shh, Ihh and Dhh. (B) In situ hybridization for Smo, Hip and Gli1. The dashed red line indicates the border between mesenchyme and epithelium. (C) X-gal staining of the esophagus of Gli1-CreERT2 x Rosa26-Stopβ/β-LacZ mice induced with tamoxifen. Arrows in the right panel indicate cells that express LacZ. Original Magnifications: 400x.
**Figure 2.** Effective recombination of *Ptch1* upon injections with tamoxifen. (A) Recombination efficiency of exons 8 and 9 of *Ptch1* and (B) quantitative RT-PCRs for Hh targets *Gli1*, *Hhip* and *Ptch1* on control and *Ptch1* mutant mice. (C) In situ hybridization for Hh target *Gli1* in control mice (left panel) and *Ptch1* mutant mice (right panel). Original magnification: 400x.

**Figure 3.** Loss of polarity and impaired maturation upon increased Hh signaling. (A) H&E stainings of *Ptch1* mutant mice and (B) K5tTA- *TreGli1* mice, both with a control shown in the left panel. Closed arrows in the left panels indicate the keratohyalin granules, which are lost in the mutant mice. Open arrows in the middle panels indicate parakeratosis. (C) Electron microscopy of a control and *Ptch1* mutant mouse. The left two panels show the basal layer, the right two panels show the suprabasal layers of the esophageal epithelium. Original magnifications (A,B): 400x, (C): 2600x.
Figure 4. Increased proliferation of precursor cells and retention of differentiating cells in the epithelial layer. (A) Immunohistochemistry for Pcna in control mice (left panel) and mutant mice (right panel). (B) Counting of Pcna positive cells per 500 μm in the basal epithelial layer and each layer above. (C) Immunohistochemical staining for BrdU positive cells 48 hours after a BrdU pulse. (D) Counting of BrdU positive cells per transverse section. (E) Immunofluorescent double staining of Ki67 (red) and BrdU (green) in the BrdU pulsed mutant mice. Original magnifications: 400x (A,E), 200x (C).
**Figure 5.** Increased expression of basal cell markers and decreased expression of differentiation markers of the esophageal epithelium of Ptch1^{fl/fl} mutant mice. (A) Immunohistochemical staining for p63, a basal cell marker (left). Counting of p63 positive cells per 100 μm in control and Ptch1^{fl/fl} mutant mice (middle) and quantitative RT-PCR for p63 (right). (B) Immunohistochemical staining and quantitative RT-PCR for keratin 14, another basal cell marker. (C-E) Immunohistochemical staining for the differentiation markers loricrin (C), keratin 13 (D) and involucrin (E). The red arrow in D indicates the presence of an unfragmented nucleus in the keratin layer ('parakeratosis'). Original magnifications: 400x (A).

**Figure 6.** (A) Immunohistochemistry and (B) quantitative RT-PCR for Klf4. Original magnification: 400x.
Figure 7. (A) Quantitative RT-PCR demonstrates significant upregulation of Bmp7 in the Ptch1fl/fl mutant mice. In situ hybridization shows that Bmp7 is expressed by the basal cells of the epithelium. (B) Immunohistochemistry for mediators of Bmp signaling phospho-Smad1,5,8 shows up-regulation in the Ptch1fl/fl mutant mice. (C) Treatment of Het1A cells with recombinant BMP7 demonstrates up-regulation of BMP targets ID2 and ID4 and of differentiation marker involucrin. No effect was observed on expression of KLF4. Original magnifications: 400x.
Supplementary Figure 1. Expression of Shh and Hh targets Gli1 and Hhip in the mouse embryo (E13.5) examined by in situ hybridization. During development Gli1 and Hhip are expressed by the mesenchyme. The dashed red line indicates the border between epithelium and mesenchyme in the esophagus. Original magnifications: 200x.

Figure 2. Histological parameters for inflammation
(A) Scoring histological parameters for inflammation showed that edema, crypt loss and mononuclear cell infiltration were significantly reduced in the PI3K-kinase-dead mice ($P=0.0066$, $P=0.046$ and $0.0071$ respectively); the amount of granulocytes, fibrosis and ulceration were reduced, but not significantly ($P=0.72$, $P=0.66$ and $P=0.27$ respectively). (n=10 per group). (B) H&E staining of the colons of both wild-type and mutant mice shows that inflammation was less prominent in the PI3K-kinase-dead tan in wild type mice. The mutant mice have less edema (left panels), reduced crypt loss and a decreased amount of inflammatory cells (right panels).
Figure 5. Absence of functional PI3Kγ prevents recruitment of new leukocytes in response to treatment with DSS

(A,B) Immunohistochemical stainings for F4/80, a macrophage marker, and CD3, a T cell marker, in unchallenged mice and mice treated with DSS. (A) The strong influx of macrophages that is observed in wild type mice is completely abrogated in PI3Kγ-kinase-dead mice (P<0.0001, n=10 per group). (B) Recruitment of T cells was similarly completely absent in the PI3Kγ-kinase-dead mice (P<0.0001, n=10 per group). Immunological staining for B220 showed staining of B-cells in the follicles (C left panel), but very little cells in the rest of the colonic lamina propria (C right panel). (D) Immunological staining for Ly6G showed absence of neutrophils, using a pulmonary infiltrate as positive control (insert in D). Original magnifications: 100x (A,B), 40x (C left,D) and 200x (C right).