Endodermal Hedgehog signals modulate Notch pathway activity in the developing digestive tract mesenchyme

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SUMMARY
The digestive tract epithelium and its adjoining mesenchyme undergo coordinated patterning and growth during development. The signals they exchange in the process are not fully characterized but include ligands of the Hedgehog (Hh) family, which originate in the epithelium and are necessary for mesenchymal cells to expand in number and drive elongation of the developing gut tube. The Notch signaling pathway has known requirements in fetal and adult intestinal epithelial progenitors. We detected Notch pathway activity in the embryonic gut mesenchyme and used conditional knockout mice to study its function. Selective disruption of the Notch effector gene RBP-Jk (Rbpj) in the mesenchyme caused progressive loss of subepithelial fibroblasts and abbreviated gut length, revealing an unexpected requirement in this compartment. Surprisingly, constitutive Notch activity also induced rapid mesenchymal cell loss and impaired organogenesis, probably resulting from increased cell death and suggesting the need for a delicate balance in Notch signaling. Because digestive tract anomalies in mouse embryos with excess Notch activity phenocopy the absence of Hh signaling, we postulated that endodermal Hh restrains mesenchymal Notch pathway activity. Indeed, Hh-deficient embryos showed Notch overactivity in their defective gut mesenchyme and exposure to recombinant sonic hedgehog could override Notch-induced death of cultured fetal gut mesenchymal cells. These results reveal unexpected interactions between prominent signals in gastrointestinal development and provide a coherent explanation for Hh requirements in mesenchymal cell survival and organ growth.

KEY WORDS: Hedgehog, Intestine, Stomach, Endoderm, Mesoderm, Notch, Hes1, Mouse

INTRODUCTION
The union of gut endoderm and splanchnic mesoderm generates the digestive tract and its derivatives – the liver, pancreas and bile ducts (Wells and Melton, 1999). Beneath the specialized epithelia in these organs, cells of mesodermal origin produce fibroblasts and smooth muscle and recruit blood vessels and neurons (McLin et al., 2009). Endoderm-mesenchyme interactions are necessary to specify the epithelia (Golosow and Grobstein, 1962; Roberts, 2000) and the mesenchyme is required for growth and morphogenesis of the tubal gut. The sequela of congenital or acquired human short bowel syndromes underscore the importance of intestinal elongation in particular (Weaver et al., 1991). The vertebrate digestive tract hence serves as a significant model for the study of tissue interactions in organogenesis.

Recent investigation has begun to elucidate the roles of selected signaling pathways in gastrointestinal development. For example, bone morphogenetic protein 4 (Bmp4) signals from the lamina propria mesenchyme to control intestinal crypt formation (Haramis et al., 2004). Conversely, platelet-derived growth factor alpha (Pdgfa) in the epithelium signals through receptors in the mesenchyme to regulate villus morphogenesis (Karlsson et al., 2000) and epithelial secretion of fibroblast growth factor 9 (Fgfl9) drives mesenchymal cell proliferation and myofibroblast differentiation (Geske et al., 2008). Because two Hedgehog (Hh) family ligands, sonic hedgehog (Shh) and Indian hedgehog (Ihh), are highly expressed in embryonic mouse gut endoderm, suggesting possibly redundant functions (Ramalho-Santos et al., 2000), we recently studied the consequences of the effective absence of all endodermal Hh signaling in Shh–/–;Ihh–/– mice. Compound mutant embryos showed profoundly reduced stomach and intestinal size, reflecting rapid and nearly complete loss of mesenchymal cells; consistent with a stimulatory role for Hh, cultured fetal gut mesenchymal cells replicated briskly in response to exogenous Shh (Mao et al., 2010). Although the roles of individual signaling pathways are gradually coming into sharper focus, we know little about how they interact with one another or about the basis for Hh dependence.

Notch signaling controls diverse aspects of development (Artavanis-Tsakonas et al., 1999). In the intestine, genetic analyses of zebrafish and mouse mutants reveal a requirement in cell expansion and proper lineage allocation of epithelial progenitors (Crosnier et al., 2005; Fre et al., 2005; van Es et al., 2005). Notch signaling also regulates proliferation and differentiation of the neural crest-derived enteric nervous system (Okamura and Saga, 2008; Taylor et al., 2007). Because Notch signaling is used repetitively in development and homeostasis, and might therefore have complex functions in digestive tract organogenesis, we examined a possible role in mesenchymal cells. Here, we show that Notch signaling occurs in embryonic intestinal and stomach mesenchymal fibroblasts, which are progressively depleted in its absence. Surprisingly, excessive Notch signaling through forced expression of the Notch intracellular domain (NICD) in the same cells had a similar, but exaggerated, effect, with profound and rapid loss of subepithelial mesenchyme and resulting truncation of the digestive tract.
tract. This abnormality phenocopies the complete loss of endodermal Hh signaling, and cultured mouse fetal stomach mesenchymal cells are exquisitely sensitive to the expression of NICD. Thus, the developing gut mesenchyme requires a fine balance in Notch pathway activity, which is required to achieve proper organ size but is lethal when uncontrolled. The unexpected similarity between mutant NICD-expressing and Hh null mutant mouse phenotypes led us to postulate that one crucial Hh function in gut development is to restrain the extent of Notch signaling activity. Indeed, recombinant Shh rescued cells from NICD-induced death and, conversely, Shh;Ihh double-null embryos showed evidence of increased Notch pathway activity in the defective gut mesenchyme. These results uncover an important and novel interaction between major signaling pathways in mesenchymal cells that control the development of the digestive tract.

**MATERIALS AND METHODS**

**Mice**

Bapx1Cre mice (Verzi et al., 2009) were a generous gift from W. Zimmer and RBP-JCre mice (Han et al., 2002) were generously provided by S. Artavanis Tsakonas with permission from T. Honjo. Rosa26Fl/+ reporter mice (Srinivas et al., 2001) and Rosa26RosaCre reporter mice (Murtaugh et al., 2003) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Shh1Cre;IhhFl mice (Han et al., 2002) were generously provided by S. Artavanis Tsakonas with permission from T. Honjo. Pax6-Cre transgenic mice were described previously (Rowan et al., 2004). Pbx2;Ihh knock-in mice (Murtaugh et al., 2003) and ShhCre/+;Ihh+/– reporter mice (Verzi et al., 2009) were a generous gift from W. Zimmer and S. Artavanis Tsakonas, respectively. To obtain ShhCre/Fl;Ihh–/Fl mice, the ShhCre/+;Ihh+/– animals were mated with Rosa26Nicd Fl/Fl males (Han et al., 2002) and Rosa26Nicd Fl/Fl females (Mao et al., 2010). Littermates lacking Cre expression and heterozygous mice served as controls for each experiment. Animals were housed and handled according to protocols approved by institutional committees.

**Immunohistochemistry, histology and in situ hybridization**

Tissues were fixed overnight in 4% parafomaldehyde at 4°C, dehydrated, embedded in paraffin, and sectioned at 5 μm. Antigens were retrieved in 10 mM Na citrate buffer (pH 6.0) and endogenous peroxidase activity blocked in methanol and 3% H2O2. Frozen tissues were embedded in OCT compound (Tissue-Tek) and sectioned at 9 μm. After blocking with 5% fetal bovine serum, samples were incubated with the following antibodies: Atp4b (2B6, 1:1000, MBL, Billerica, MA, USA), smooth muscle actin (1A4, 1:3000, Biogenex, San Ramon, CA, USA), β-tubulin (TuJ1, 1:200, BD Pharmingen, Franklin Lakes, NJ, USA), p63 (1:2000, Neomarkers, Fremont, CA, USA), Ki67 (1:2000, Vector Labs, Burlingame, CA, USA), and GFP (JL-8, 1:200, Clontech, Mountain View, CA, USA), phospho-histone H3 (1:500, Millipore, Billerica, MA, USA), and Hes1 (1:1000, gift of Dr Nadean Brown, University of Cincinnati, OH, USA), phospho-histone H3 (1:500, Millipore, Billerica, MA, USA), and Hes1 (1:1000, gift of Dr Nadean Brown, University of Cincinnati, OH, USA), and Hes1 (1:1000, gift of Dr Nadean Brown, University of Cincinnati, OH, USA). Samples were washed and subsequently incubated with biotin-conjugated anti-mouse or anti-rabbit IgG (1:300, Vector Labs). Color reactions were developed with Vectastain avidin-biotin complex ABC kit (Vector Labs) and diaminobenzidine substrate (Sigma-Aldrich, St Louis, MO, USA). Immunohistochemistry, histology and in situ hybridization were performed according to protocols approved by institutional committees.

**Fig. 1. Expression of Notch receptors and ligands and their target gene Hes1 in mouse embryonic gut mesenchyme.**

(A) E12.5 stomach endoderm (SE) and mesenchyme (SM) were separated and analyzed by conventional (gel electrophoresis bands) and qRT-PCR (bar graph). Expression of Notch2 and Notch3 (but not Notch1) transcripts is roughly equivalent in the two compartments. Compartment-specific expression of Bapx1 and Shh verifies tissue separation and Hes1 signal reveals greater Notch signaling in endoderm than in mesenchyme. Normalized average Ct values from each sample, assayed in triplicate, are shown below the qRT-PCR bars, where SM transcript levels are expressed in relation to the SE level, normalized to 1.0. (B) qRT-PCR analysis for Notch ligands in E12.5 SE and SM. Mesenchymal expression of Jag1 and Dil3 mRNA, but not other Notch ligands, approaches that in the endoderm. qRT-PCR analyses (A,B) were performed on three independent samples; error bars indicate s.d. (C-G) In situ hybridization results for Notch3, Jag1 and Dil3 in E14.5 stomach, with sense controls for Jag1 and Dil3. Dotted lines demarcate endoderm (luminal) from mesenchyme. (H-J) Hes1 immunostaining on E15 digestive tract, including the stomach (H, the boxed area is magnified in I) and intestine (J). Besides the nascent epithelium, Hes1 appears in the mesenchymal lamina propria (arrows). Scale bars: 50 μm.
5× SSC, 2× Denhardt’s solution, 0.02% bovine serum albumin, 0.1% Tween 20, 0.25% SDS, 5 mM EDTA (pH 8.0) and 50 μg/ml yeast tRNA. After washing, slides were incubated for 90 minutes in 20% sheep serum and then overnight at 4°C with alkaline phosphatase-conjugated sheep antidigoxigenin antibody (1:3000 in PBS supplemented with 10% fetal bovine serum). Color reactions were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

Reverse transcription (RT) PCR
Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA), treated with RNase-free DNase (Ambion, Austin, TX, USA), and reverse transcribed using oligo(dT) primers. Relative transcript levels were determined by conventional and SYBR Green real-time PCR (Applied Biosystems, Carlsbad, CA, USA).

Ex vivo evaluation of Shh and NICD effects
E12.5 mouse stomach and intestine mesenchymal cells were isolated as described previously (Kim et al., 2005), seeded into 12- or 96-well dishes, and cultured in alpha-MEM containing minimal serum (Mediatech, Herndon, VA, USA). To test if N-Shh protects cells from NICD-induced cell death, cells were transfected with either GFP or NICD-GFP cDNA inserts cloned in the pCIG expression vector (Megason and McMahon, 2002) and treated with 80 nM palmitic acid-modified recombinant N-Shh (Pepinsky et al., 1998) for 3-24 hours. Cell viability and ratios of GFP+ cells to all DAPI-stained nuclei were determined under inverted fluorescence microscopy. To test the Hh-Notch interaction ex vivo, we treated endoderm-depleted E12.5 mouse stomach explants with 10 μM cyclopamine-KAAD (EMD Chemicals, San Diego, CA, USA), replenishing the compound daily for 4 days.

Fig. 2. Notch signaling is required for gut elongation and maintenance of lamina propria mesenchyme. (A) Whole-mount images of the shortened intestine in 5-day-old Bapx1+/Cre;RBP-JkJF/Fl mouse pups (right) as compared with control littermates (left). Dotted white lines mark the small intestine boundaries at the gastroduodenal and ileocolic junctions. Throughout this figure, control (CONT) refers to Bapx1+/+, RBP-JkJF/Fl, or Bapx1+/Cre;RBP-JkJF/Fl, both of which resemble the wild type. (B, C) Examples (B, C) and quantitation (C) of small bowel length in Bapx1+/Cre;RBP-JkJF/Fl and littermate controls at E14, E16 and birth. Mutant neonates, the body size of which is similar to that of control littermates (E16 example shown in B), exhibit gut shortening (red line in C, right) and reduced spleen size (B, bottom). Mean ± s.d.; n=3 for each sample. (D) Significant depletion of the mesenchymal compartment in Bapx1+/Cre;RBP-JkJF/Fl newborn intestine and stomach, with normal smooth muscle and neuronal differentiation revealed by smooth muscle actin (SMA) and neuron-specific β-tubulin immunostaining (Tuj1). Insets show higher magnification of the boxed regions. We examined seven P0 mutants and littermate controls and at least three mutant embryos and controls at each stage. Scale bars: 50 μm.
RESULTS
Notch signaling in the developing mouse gut mesenchyme

Mammals have four Notch receptors, Notch1-4, and five ligands, jagged 1/2 (Jag1/2) and delta-like 1-3 (Dll1-3) (Bray, 2006). Ligand-induced proteolysis results in translocation of the Notch intracellular domain (NICD) to the nucleus, where it associates with the transcription factor RBP-J (Rbpj – Mouse Genome Informatics) and activates target genes such as Hes1 (Kopan and Ilagan, 2009). In epithelial and mesenchymal fractions isolated from wild-type mouse E12.5 stomach, conventional and quantitative (q) RT-PCR analyses showed that Notch1 mRNA is largely endodermal, whereas Notch2 and Notch3 levels are comparable in the two fractions and Notch4 is absent from both (Fig. 1A). We also detected mesenchymal expression of Jag1 and Dll3 (Fig. 1B), and in situ hybridization confirmed expression of Notch3, Jag1 and Dll3 in mesenchymal cells (Fig. 1C-G). RT-PCR analyses suggested (Fig. 1A) and immunohistochemistry confirmed (Fig. 1H-J) that the best-characterized Notch target gene, Hes1 (Ohtsuka et al., 1999), is expressed not only in fetal stomach and intestinal epithelium but also in the adjacent mesenchyme, especially in lamina propria fibroblasts just beneath the nascent epithelium (arrows in Fig. 1I,J). These results demonstrate Notch pathway activity in the developing mammalian gut mesenchyme.

Mesenchymal Notch signaling is required for gut fibroblast proliferation and proper organ size

To determine the function of mesenchymal Notch signaling, we inactivated the pathway using a well-established Cre-dependent null RBP-Jk allele (Han et al., 2002) and Bapx1Cre (Bapx1 is also known as Nkx3.2 – Mouse Genome Informatics) mice, which express Cre recombinase throughout the intestinal and distal stomach mesenchyme after E9.5 (Verzi et al., 2009). Staining of Bapx1Cre;Rosa26Lox-STOP-Lox-YFP organs for YFP and smooth muscle α-actin (Acta2 – Mouse Genome Informatics) or neuron-specific β-tubulin (TuJ1) verified Cre expression in the precursors to all lamina propria fibroblasts and smooth muscle cells but not in enteric neurons or any epithelial cells (see Fig. S1A in the supplementary material). Mesenchyme-restricted ablation of Notch function in Bapx1Cre;RBP-JkFl/Fl mice was lethal in 7 out of 8 pups by 1 week of age. Intestinal length was significantly reduced in 5-day-old pups (Fig. 2A), hinting at malnutrition as the cause of death. This shortening reflected a developmental defect because although overall embryo dimensions were similar among wild-type, Bapx1+/Cre, Bapx1Cre;RBP-Jk+/Fl and Bapx1Cre;RBP-JkFl/Fl littermates (Fig. 2B), the intestines in E14, E16 and newborn Notch-deficient mutants were 20-30% shorter on average (Fig. 2C). The spleen, another site of Bapx1-Cre expression (Verzi et al., 2009), was also reduced in size (Fig. 2B).

Fig. 3. Effect of Notch inactivity on lamina propria mesenchyme. (A) Lack of Notch signaling selectively depletes fibroblasts in the space (red bars) between the subepithelial basement membrane and circular smooth muscle, as revealed in SMA immunomicrographs of embryonic mouse intestine. To the right, measured mesenchymal thickness is represented as a fraction (%) of the average in littermate controls, which included wild-type, Bapx1Cre and Bapx1Cre;RBP-JkFl/Fl embryos. Error bars indicate s.d.; n≥3 for each sample. Int, intestine; St, stomach. (B) Markedly reduced proliferation of mesenchymal fibroblasts, as detected with Ki67 immunostaining of newborn intestines and phospho-histone H3 immunostaining at E16 (see Fig. S1D in the supplementary material). Boxed areas are magnified to the right and dotted lines delineate the epithelium-mesenchyme border. (C,D) Histology (C) and SMA immunohistochemistry (D) show that lamina propria mesenchyme is also depleted in mutant mouse stomachs. The light brown signal in D represents subepithelial myofibroblasts. Seven P0 mutant embryos with littermate controls were analyzed and at least three mutant embryos with controls were examined for other stages. Scale bars: 50 μm.
As a likely explanation for limited intestinal growth, lamina propria fibroblasts between the basement membrane and smooth muscle were notably deficient in Bapx1+/Cre;RBP-JκFl/Fl intestines, whereas the numbers of SMA+ smooth muscle cells and Tuj1+ neurons were unaffected (Fig. 2D; see Fig. S1B and Fig. S2A,E in the supplementary material). Close inspection of the tissue confirmed selective diminution of subepithelial fibroblasts and a corresponding reduction in mesenchymal thickness in the developing gut (Fig. 3A); these defects were evident in both the proximal and distal small intestine but less so in the colon (see Fig. S2 in the supplementary material). To determine the basis for this selective depletion, we examined cell replication. Compared with control RBP-Jκ+/+ fibroblasts, the fraction of mutant cells expressing the replication markers Ki67 (Fig. 3B; see Fig. S2B,E in the supplementary material) or phospho-histone H3 (see Fig. S1C in the supplementary material) was markedly reduced in embryos and neonates. Only subepithelial fibroblasts, in which Notch receptors and Hes1 are expressed (Fig. 1), showed reduced proliferation, with an insignificant increase in apoptosis at E14, E16 and P0 (data not shown). Villi in the mutant intestine were stunted (see Fig. S1B in the supplementary material; Fig. 2D), but epithelial differentiation, including the proportion of goblet and enteroendocrine cells, was overtly normal (see Fig. S2C,D in the supplementary material).

In the proximal domain of Bapx1-Cre expression, RBP-Jκ deficiency also resulted in considerable depletion and reduced proliferation of lamina propria fibroblasts in the gastric antrum and pylorus (Fig. 3A,C,D). Stomach glands often failed to organize into characteristic tall, pitted units (Fig. 3C). As Cre-mediated recombination in Bapx1Cre mice is restricted to the mesenchyme (see Fig. S1A in the supplementary material), these epithelial defects must reflect the response to a primary mesenchymal abnormality resulting from Notch pathway inactivity.

**Gain of Notch signaling in the gut mesenchyme also causes mesenchymal cell attrition**

To test the effect of unregulated Notch activity in the gut mesenchyme, we crossed Bapx1Cre and Rosa26Lox-STOP-Lox-Nicd (Rosa26Nicd) (Murtaugh et al., 2003) to produce mice expressing NICD1, the active intracellular domain of Notch1 (Kopan and Ilagan, 2009), in the Bapx1 domain. The resulting ligand-independent Notch activation caused even more profound shortening...
of the digestive tract and abnormal tissue architecture than did the abolition of Notch activity. The esophagus and proximal stomach, which lack Bapx1 expression (Verzi et al., 2009), developed normally, but the remaining alimentary canal was markedly abbreviated (Fig. 4A,B). The wall of the shortened intestine was asymmetric, with intact smooth muscle and neuronal differentiation on one side, the absence of smooth muscle and an excess of enteric neurons on the other, and connective tissue bands bridging the two sides (Fig. 4C,D). The mucosa was also disorganized (Fig. 4B,C), presumably in response to a primary mesenchymal defect.

Marked truncation of the digestive tract is best explained by a paucity of mesenchymal fibroblasts, which drive gut growth and morphogenesis. However, we did not observe a significant reduction in the thickness of the lamina propria, which suggests that the remaining mesenchymal cells might have escaped Cre-mediated recombination. As the Rosa26(Nicd) allele expresses GFP from an internal ribosome entry site (Murtough et al., 2003), it is possible to distinguish the progeny of Bapx1-Cre-nonexpressing from recombined Cre-expressing cells. Indeed, the distal stomach and intestinal mesenchyme in Bapx1(Cre);Rosa26(Nicd) embryos carried innumerable GFP-expressing cells at E10 but completely lacked GFP expression by E14 (Fig. 4E,F), indicating cell replacement. Furthermore, small patches of mucosal cells at the stomach-intestine junction expressed the proximal stomach markers p63 (Trp63 – Mouse Genome Informatics) and Atp4b, suggesting that some neighboring Cre-deficient mesenchymal cells might also compensate for loss of NICD-expressing cells (see Fig. S3 in the supplementary material). To investigate the basis for the pronounced mesenchymal defect in Bapx1(Cre);Rosa26(Nicd) embryos, we used K167 and caspase 3 immunostaining to examine cell proliferation and death at E10, E11, E13 and E16. Proliferation was sustained at all stages, and even moderately increased in Cre-deficient mesenchymal cells in older embryos; mesenchymal cell apoptosis was evident at E13 (Fig. 4G-J; see Fig. S4 in the supplementary material). These data show that constitutive Notch activity results in death of gut mesenchymal fibroblasts.

To assess the effects of Notch pathway overactivity in another animal model, we crossed Rosa26(Nicd) mice with Pax6-Cre transgenic mice, which express Cre under the control of a 3.9 kb Pax6 upstream element. Pax6-Cre;Rosa26(Nicd) mice faithfully recapitulated endogenous Pax6 expression (Rowan et al., 2008) in the eye and pancreas. Two independent transgenic lines also expressed GFP in the stomach mesenchyme, initially in a few cells at E11 (see Fig. S5 in the supplementary material) and then in the full thickness of the mesenchyme by E14 (Fig. 5A; see Fig. S5 in the supplementary material); this distribution thus overlaps that of Bapx1-Cre but occurs later in development. Stomach shape and histology in Pax6-Cre;Rosa26(Nicd) mice from both Pax6-Cre transgenic lines were normal early in the course of transgene expression (Fig. 5B); thereafter, mesenchymal cells were rapidly lost and stomach mass failed to match the growth of other organs. The stomach wall became progressively thinner, allowing the lumen to inflate (Fig. 5C), and stretched to its limit, the wall often collapsed into the lumen (Fig. 5D). By E18.5 the stomach contained little mesenchyme and the epithelium was closely apposed to the mesogastrium; in the absence of mesenchymal support, the stomach epithelium was hypoplastic and undifferentiated (Fig. 5D). Outside the domain of Pax6-Cre transgene expression, the digestive tract developed normally. Analysis of Pax6-Cre;Rosa26(Nicd) stomachs at E14.5 did not reveal a deficit in cell proliferation (Fig. 5G,H), but showed significantly more apoptotic cells than in littermate controls (Fig. 5E,F).

Thus, two different Rosa26(Nicd) mouse strains demonstrate the profound effects of unregulated Notch signaling on embryonic gut mesenchyme. Together with the findings in Bapx1(Cre);RBP-Jκ(f/f) mice, these results indicate that alimentary canal fibroblasts are exquisitely sensitive to the level of Notch signaling, failing to survive and to contribute to organ growth when Notch activity is either absent or constitutive.
Excess Notch signaling in developing stomach mesenchyme phenocopies total Hh loss and Shh counters the effects of Notch overactivity

The consequences of Notch pathway activation in the stomach mesenchyme phenocopy defects we observed in mice lacking both of the endodermal Hh ligands, Shh and Ihh (Mao et al., 2010). In particular, stomach histomorphology in Shh;Ihh double-null embryos (ShhCreFli;IhhFli) (Mao et al., 2010) is identical to that in Pax6-Cre;Rosa26Nicd mice, which activate Notch later than the Bapx1Cre strain in the mesenchyme and avoid compensatory expansion of Cre-deficient cells. The striking and unusual concordance in loss of nearly all lamina propria fibroblasts led us to postulate that the trophic effect of endodermal Hh signaling on the adjacent mesenchyme might occur in part by restraining the level of Notch activity.

To test this hypothesis, we asked first whether Notch signaling is overactive in ShhCreFli;IhhFli embryos. Because the Shh;Ihh double-null intestine is affected severely and very early, we focused on the mutant stomach, where mesenchymal cell attrition is gradual (Mao et al., 2010). qRT-PCR analysis revealed increased mRNA levels of the Notch target genes Hes1 and Hes5 (Ohitsuka et al., 1999) in the Shh;Ihh double-mutant stomach, without increases in Notch2 or Notch3 levels; total Notch1 expression was reduced, a likely secondary effect of mesenchymal depletion on the overlying epithelium (Fig. 6A). qRT-PCR analyses also showed reduced Dll1 and Dll3 mRNA levels in Shh;Ihh double-mutant stomachs (Fig. 6B), suggesting that the Notch pathway is activated through mechanisms distal to receptor and ligand expression. As the Notch pathway signals in both endoderm and mesenchyme (Fig. 1), Hes1 mRNA levels in the whole organ represent the sum of both compartments; thus, the increase in Notch target gene expression in the mesenchyme is probably even greater than these data represent. RNA in situ hybridization and immunohistochemistry confirmed excess Hes1 expression in ShhCreFli;IhhFli stomach mesenchyme (Fig. 6D-G). Second, we cultured wild-type fetal mouse stomach explants in the presence of the Hh antagonist cyclopamine (Incardona et al., 1998). Vehicle-treated explants expressed the Hh target transcript Hhip and small amounts of Hes1 mRNA, as expected, whereas cyclopamine inhibited Hhip expression and elevated Hes1 mRNA levels (Fig. 6C). Taken together, these results reveal increased Notch pathway activity when Hh signaling is curtailed.

To test the hypothesis further in a rigorous in vitro model, we forced NICD1 and GFP expression simultaneously in cultured primary fetal mouse stomach mesenchymal cells and followed the fate of transfected, GFP+ cells. Consistent with the results of NICD1 expression in vivo, NICD1 induced rapid loss of GFP-expressing cells (Fig. 7A). Cell attrition was evident by 6 hours and no more than 20% of GFP+ cells were present by 24 hours, whereas untransfected cells or those transfected only with a GFP

![Fig. 6. Hh and Notch signaling interact in gut development. (A,B) Results of qRT-PCR for Notch receptor and target genes (A) and ligands (B), showing increased expression of the Hes1 and Hes5 targets in E12.5 Hh-deficient (ShhCreFli;IhhFli, DKO) stomachs as compared with wild-type (WT) controls. As the endodermal Hes7 signal is unchanged or decreased in mutant samples, changes observed in the whole organ reflect increased mesenchymal expression. Results represent independent experiments on six embryos of each genotype and are expressed as the mean ± s.d. of transcript levels normalized to the wild-type controls. (C) Mouse embryonic stomach explants were treated for 3 days with cyclopamine (Cyc) or vehicle (ethanol control, Ctl) and analyzed by RT-PCR. Decreased Hhip expression confirms inhibition of Hh signaling and increased Hes1 mRNA levels indicate Notch pathway overactivity in the cyclopamine-treated samples. (D,E) In situ hybridization to detect Hes1 mRNA in E12.5 control (D) and ShhCreFli;IhhFli (E) embryonic stomachs. Left and right images show low- and high-magnification micrographs, respectively. The increase in Hes1 expression in mutant stomachs occurs in the face of ongoing cell loss and is confined to the mesenchyme, where Hes1 mRNA is barely detected in controls (see also Fig. 1A). (F,G) Antibody staining confirms the increase in Hes1 protein in subepithelial fibroblasts in ShhCreFli;IhhFli (G) compared with control (F) embryonic stomach. Magnified views of the boxed areas are shown. The dotted lines separate endoderm from mesenchyme.](image-url)
plasmid thrived. This pace of cell loss is most compatible with a failure of cell survival, similar to the finding of increased apoptosis in mouse models of forced mesenchymal NICD expression (Fig. 4H and Fig. 5F). Exposure to recombinant active, lipid-modified Shh [N-Shh (Pepinsky et al., 1998)] protected cells from NICD-induced death, largely overriding the lethal effect of forced Notch activation (Fig. 7A). These observations collectively support the idea that gut endodermal Hh signals modulate the level of Notch pathway activity and help avoid lethality associated with excessive Notch signaling in the adjoining mesenchyme (Fig. 7B).

**DISCUSSION**

Organogenesis requires exquisite control over the timing, regional distribution and magnitude of the activities of developmental signaling pathways. The digestive tract is a good model with which to study tissue and pathway interactions in development because its anatomic origins are simple and well understood, and because interactions between the epithelial and subepithelial compartments are responsible for the emergence of form, pattern and function. Notch signaling is involved in homeostasis in many tissues and self-renewing organs, including the intestinal epithelium (Crosnier et al., 2005; Fre et al., 2005; van Es et al., 2005). Our studies uncover a complex and unexpected role for Notch signaling in the development of digestive tract mesenchyme and reveal the fine balance between insufficient and excessive Notch pathway activity in this tissue.

Notably, both loss and gain of Notch function led to a similar outcome of mesenchymal cell depletion. The significantly diminished proliferation of mesenchymal cells in Bapx1^−/Cre;RBP-Jκ^+/− mouse embryonic gut implicates a role for Notch signaling in the replication of subepithelial fibroblasts, whereas constitutive Notch activation induces the same cells to die. Thus, Notch seems to play a bipartite role common to many growth signals, promoting both cell survival and proliferation when the level of pathway activity is moderate. Notch activity and requirement are confined to the lamina propria fibroblasts that lie immediately beneath the basement membrane, in a compartment that serves as an abundant source of cells for the considerable, Notch-dependent growth and lengthening of the embryonic digestive tract. These fibroblasts reside close to the epithelial source of Hh proteins, at a distance that is compatible with the inhibitory effect of Hh signaling on Notch pathway activity. As Notch signaling is necessary to ensure sufficient mesenchymal mass for organ enlargement, a certain level of Notch pathway activity is clearly required in lamina propria fibroblasts. We can only speculate why excessive activity is lethal for the same cells. Perhaps Notch target genes are only tolerated transiently and their sustained expression is detrimental, or high Notch activity induces deleterious behaviors, such as cell migration or inappropriate differentiation, and cell suicide is a protective response. Irrespective of the underlying reasons, our results reveal the adverse consequences of inadequate or excessive Notch activity and the need for tight regulation.

The sum of our observations supports the model illustrated in Fig. 7B, whereby endoderm- or epithelium-derived Hh curtails the extent of mesenchymal Notch activity, permitting a level that supports fibroblast replication and organ growth without compromising viability. It is, however, unclear exactly how Hh signaling achieves this effect. The increased Hes1 and Hes5 expression in mouse embryos lacking Hh activity was not accompanied by increased expression of Notch receptors or ligands. Dll3 mRNA levels were reduced by at least 50% in the whole stomach and could potentially block Notch signaling (Ladi et al., 2005), but Dll1 levels were comparably reduced, suggesting that Hh modulates pathway activity at a step distal to Notch receptor and ligand expression. Consistent with this possibility, Shh rescued cell death in fetal mouse stomach mesenchymal cells forced to express NICD. Our results hence suggest that Hh signaling provides protection from cell death by acting either at a distal step in the Notch pathway, beyond NICD release, or by modulating the levels or activities of Notch target genes that threaten mesenchymal cell viability. Our studies do not address whether Notch pathway activity in lamina propria fibroblasts in turn influences the level of epithelial Hh secretion, which is certainly possible.

In vertebrate digestive tract development, the role of mesoderm-derived mesenchyme has historically been emphasized for its permissive and inductive functions, especially in regional patterning but also in epithelial morphogenesis and differentiation (Kedingter et al., 1998). By contrast, the role of the endoderm-derived epithelium in subepithelial cell development is less well appreciated. Fgf9 secretion from the epithelium was recently shown to influence mesenchymal proliferation and myofibroblast differentiation (Geske et al., 2008), and we demonstrated the profound dependence of mesenchymal cell survival and replication throughout the gut tube on endodermal Hh signals (Mao et al., 2010). The present observations advance our understanding of Hh functions in this context, revealing it as a means to modulate Notch pathway activity and possibly to convey information to lamina propria fibroblasts regarding their position along the radial axis of the developing gut. Our observations also reaffirm that proper mesenchymal mass is necessary for the maintenance and maturation of the overlying digestive epithelium and emphasize the bidirectional flow of information in gastrointestinal development.

These data add to the previous demonstration of functions for Bmp4 and Pdrgfa, and should stimulate further investigation of the interactions among the handful of implicated signals. Of note, unlike these factors, which can diffuse across tissues to varying degrees, Notch signaling requires cell-cell contact because Notch ligands are transmembrane proteins; accordingly, the Notch pathway has little potential for regulation across distances. To be able to elicit the activity that we detect in nearly all lamina propria
fibroblasts, we presume that Notch ligands and receptors are expressed on all cells, as suggested by our in situ hybridization data. In this light, the local consequences might differ from the lateral inhibition activity classically ascribed to Notch signaling in development (Chitnis, 1995). However, because gut subepithelial fibroblasts appear uniform in morphology we have little appreciation for their potential functional diversity and it is certainly possible that Notch signaling has a role in generating functionally distinct subpopulations. This would present an additional reason for the tight regulation of Notch activity levels and intolerance of excessive pathway activity.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
T.-H.K., B.-M.K., J.M. and S.R. performed the research; all authors interpreted data; T.-H.K. and R.A.S. drafted the manuscript.

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