Direct regulation of intestinal fate by Notch

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The signals that maintain the proper balance between adult intestinal cell types are poorly understood. Loss-of-function studies have implicated the Notch pathway in the regulation of intestinal fate during development. However, it is unknown whether Notch has a role in maintaining the balance of different cell types in the adult intestine and whether it acts reversibly. To determine whether Notch has a direct effect on intestinal development and adult intestinal cell turnover, we have used a gain-of-function approach to activate Notch. Ectopic Notch signaling in adult intestinal progenitor cells leads to a bias against secretory fates, whereas ectopic Notch activation in the embryonic foregut results in reversible defects in villus morphogenesis and loss of the proliferative progenitor compartment. We conclude that Notch regulates adult intestinal development by controlling the balance between secretory and absorptive cell types. In the embryo, Notch activation perturbs morphogenesis, possibly through effects on stem or progenitor cells.

differentiation | intestine | lineage | stem cells

Tissues are generated by two distinct mechanisms: duplication of existing cells or growth and differentiation of self-renewing stem or progenitor cells. Whereas the latter mechanism predominates in embryonic development, only a subset of adult tissues (notably the intestine, blood, and skin) are known to be maintained through the activity of stem cells. It is unknown whether cell fate decisions in these adult tissues are determined by the same mechanisms that are used in the embryo. The spatial organization of the intestinal epithelium into villi and crypts makes it ideal for studying differentiation. The four differentiated cell types of the intestine (enterocytes, enteroendocrine cells, goblet cells, and Paneth cells) are derived from the stepwise differentiation of crypt stem cells into a transient amplifying crypt "proliferative compartment" and subsequent differentiation into more specialized villus cells. Three of these cell types (endocrine, goblet, and Paneth cells) have been functionally grouped as the "secretory lineage" because their formation depends upon the Math1 transcription factor (1). Wnt signals play a critical role in intestinal development by regulating the pool of undifferentiated stem/progenitor cells. Inactivation of the gene encoding Tcf4, a downstream mediator of Wnt signals, or intestinal misexpression of the soluble Wnt inhibitor Dickkopf1, results in the loss of the proliferative compartment of the intestinal crypts (2, 3). Wnt signals are integrated with signals from bone morphogenic proteins (BMPs), which converge on the phosphoinositide-3 (PI3) and Akt kinases to maintain the stem cell compartment (4).

The Notch pathway acts in a variety of embryonic tissues to shape cellular repertoires by inducing or inhibiting the outcome of cell fate decisions in a context-dependent manner (5). During adult hematopoiesis, Notch has the dual activity of maintaining the undifferentiated state of hematopoietic stem cells (HSCs) while biasing committed progenitors toward certain fates (6). Notch may also play a role in intestinal development. Multiple Notch receptors and ligands are expressed in the intestine (7, 8), and three Notch-regulated genes have intestinal phenotypes. Specifically, inactivation of *Neurogenin3* (*Ngn3*) or *Hes1* alters endocrine differentiation (9–11), whereas inactivation of *Math1* results in the absence of all secretory cell types (1). However, these studies implicate Notch only indirectly in intestinal development, and do not address the question of mechanism.

Several important questions remain unanswered. First, do mutations in *Ngn3*, *Hes1*, and *Math1* interrupt a Notch signaling cascade, or are other signals such as Wnt signals involved? Indeed, there is evidence that Math1 is regulated by Wnt signals, because overexpression of Dickkopf1 in the intestine leads to loss of Math1 expression (3). Second, what mechanism might Notch use to regulate intestinal development, and is such regulation reversible or irreversible? In other systems, Notch regulates cell fate through a variety of mechanisms, including inhibition of progenitor cell differentiation (5), or loss of multipotency with accelerated differentiation (12). Third, does Notch function in the adult intestine?

We have developed reagents that permit activation of the Notch pathway in the embryonic foregut and adult intestine *in vivo* to address some of these questions through gain-of-function. We report that, in the embryo, Notch signals cause a reversible arrest of intestinal morphogenesis, with a depletion of proliferative progenitor cells. By contrast, Notch signaling in adult progenitors biases cell fate decisions without altering morphogenesis. These findings parallel observations from other adult tissues, in which Notch regulates differentiation by exerting different activities early and late in development.

Materials and Methods

Animals. Mice were maintained under specific-pathogen-free conditions on a mixed genetic background with the ICR strain. For experiments involving conditional gene expression *in vivo*, timed pregnancies were determined by checking for vaginal plugs [day of plugging was regarded as embryonic day (E) 0.5], and tetracycline (1 mg/ml with 3% sucrose) was added or removed from the drinking water at the indicated time(s).

To create the *tetO-NotchIC* strain, the intracellular portion of Notch1 (amino acids 1749–2293) was cloned into the ClaI/EcoRV sites of the pTet-Splice vector (Invitrogen). Purified insert DNA was injected into B6CBAF1 pronuclei, and founder lines were generated. These were tested for transgene activation by using two different tetracycline transactivator (tTA) "driver" lines. To minimize the impact of genetic background effects, all experiments involving conditional transgene activation were conducted by using two *tetO-NotchIC* stud males. The other mouse strains used have been described: *Pdx1-tTA* (13), *Fabpl*$^{-596\ \text{to}\ +21}$*Cre* (14), *Z/AP* (15), *Pdx1-Cre* (16), and *Rosa-NotchIC* (17).

***In Situ* Hybridization and Immunohistochemistry.** *In situ* hybridization was performed by using digoxigenin-labeled riboprobes for *conductin/axin2* (I.M.A.G.E. Consortium) and *NotchIC* (using a subcloned fragment from the *NotchIC* cDNA). Paraffin sections

Abbreviations: E, embryonic day; HPAP, human placental alkaline phosphatase; tTA, tetracycline transactivator; H&E, hematoxylin/eosin.

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were hybridized overnight at 50°C–65°C; probe detection was performed by using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) and allowed to develop for 72 h.

For immunohistochemistry, wax sections were dehydrated, endogenous peroxidases were inactivated, and sections were blocked with 2% donkey serum in PBST (phosphate-buffered saline, 0.1% Tween-20). Hes1, Nkx6.1, Pdx1, and Ki67 immunostaining were performed by using antigen retrieval with citrate buffer (pH 6). Antibodies were used at the following concentrations: rabbit anti-chromogranin A/B (1:100, RDI), rabbit anti-gastrin (1:500, DAKO), rabbit anti-Nkx6.1 (1:2000; a gift from O. Madsen); guinea pig anti-Pdx1 (1:500, a gift from C. Wright), and rabbit anti-Hes1 (1:750, a gift from T. Sudo). Following incubation with the primary antibody overnight at 4°C, slides were washed with PBST or auto buffer (Fisher), incubated with an appropriate biotinylated secondary antibody (Jackson ImmunoResearch) and developed by using ABC and DAB detection reagents (Vector Laboratories).

Tissue Staining. In pilot experiments, a variety of reporters were used to mark cells that had undergone recombination from the *Fabpl-Cre* transgene, including *Z/AP*, *Rosa26-lacZ*, and the internal ribosomal entry site-GFP (IRES-GFP) contained within the Rosa-NotchIC transgene. Of these, the *Z/AP* reporter gave the most consistent results and was therefore used for all subsequent experiments. For human placental alkaline phosphatase (HPAP) staining, small intestines of *Fabpl-Cre; Z/AP* mice were fixed for 1–2 h in zinc formalin, and fragments were embedded in paraffin and sectioned. Following rehydration, endogenous alkaline phosphatases were heat inactivated at 70°C for 30 min. HPAP was developed by using BCIP (5-bromo-4-chloro-3-indolyl phosphate, 0.17 mg/ml) and NBT (nitroblue tetrazolium, 0.34 mg/ml) in NTM buffer (100 mM NaCl/100 mM Tris, pH 9.5/5 M $MgCl_2$) for 15 min at 50°C. Suitable slides were then subjected to immunohistochemistry for chromogranin A/B or staining with 1% alcian blue 8GX (Sigma) in 3% glacial acetic acid for 30 min at room temperature, followed by counterstaining with nuclear fast red (Vector Laboratories). TUNEL staining was performed by using the DeadEnd apoptosis detection system (Promega). BrdUrd labeling was performed by injecting pregnant animals with BrdUrd 2 h before killing and was detected by using a BrdUrd detection kit (Amersham Pharmacia).

Results and Discussion

Labeling Adult Intestinal Progenitor Cells and Misexpression of Notch. Lineage analysis of adult intestine has revealed that unipotent and multipotent progenitors coexist in the crypts and have different half-lives (18). Although the transcriptional "signature" of cells that reside in the crypts has been partially characterized (19), there are only a few markers of intestinal progenitor cells (4). The fortuitous discovery that the promoter of the rat liver fatty acid-binding protein (L-FABP) gene drives the expression of transgenes in intestinal progenitor cells *in vivo* (20) makes it possible to manipulate this compartment genetically. In particular, a transgenic strain in which expression of Cre recombinase is under the control the rat *Fabpl* promoter supports heritable recombination in multipotent intestinal progenitor cells and marks all intestinal lineages when crossed with a reporter strain (14).

To label intestinal progenitor cells and their progeny, we crossed *Fabpl-Cre* transgenic mice with mice from the *Z/AP* reporter strain (15), resulting in the heritable expression of HPAP in the proximal intestine (Fig. 1*A*). Under normal circumstances, intestinal division and differentiation occurs in an orderly fashion from the crypt to the villus. Because expression of the *Fabpl-Cre* transgene is mosaic, only a fraction of crypt progenitor cells (and their progeny) were labeled. As predicted, clones of $HPAP^+$ cells were seen within segments of villi (Fig. 1 *B* and *C*), reflecting the derivation of each of these clones from a single crypt stem/progenitor cell (21). To ensure that the *Fabpl-Cre* transgene marked cells with multilineage potential, we stained $HPAP^+$ villi with alcian blue to mark goblet cells (Fig. 1*D*) or antibodies that recognize chromogranin A/B to mark enteroendocrine cells (Fig. 1*F*). Labeled segments contained enterocytes, goblet cells, and enteroendocrine cells; goblet cells composed ≈7% of the labeled cells, whereas endocrine cells composed ≈2%. This strain of *Fabpl-Cre* was inefficient at mediating recombination in the small intestine, making it difficult to find labeled crypts; this limitation precluded the quantitative comparison of cycling cells and Paneth cells within the crypts.

We next tested the effect of expressing a constitutively active form of the Notch1 receptor (NotchIC) by crossing *Fabpl-Cre;Z/AP* mice to *Rosa-NotchIC* transgenic mice; the *Rosa-NotchIC* strain permits conditional expression of NotchIC in cells expressing Cre recombinase (17). Villus segments that exhibited HPAP activity, indicating prior Cre activity, were histologically normal, but contained relatively few goblet or enteroendocrine cells (Fig. 1 *E* and *G*). Enteroendocrine and goblet cells in $HPAP^+$ villi were compared quantitatively with their counterparts in adjacent $HPAP^-$ villi. Both types of secretory cells were reduced in frequency by 5- to 7-fold in $HPAP^+$ villus segments compared with either of the control villus segments (Fig. 2 and Table 1, which is published as supporting information on the PNAS web site).

Viewed in the context of the loss-of-function phenotypes of *Ngn3*, *Hes1*, and *Math1*, these results demonstrate that Notch directly regulates adult intestinal differentiation *in vivo* by directing cells toward an enterocyte fate at the expense of secretory fates. These results are consistent with prior studies using γ-secretase inhibitors, which nonspecifically block Notch signaling by inhibiting the protease that activates the receptor and cause goblet cell hyperplasia (22–24). Villus segments exhibiting evidence of prior Cre activity have a relative, rather than absolute, reduction in secretory cells. There are two possible explanations for this finding. First, because recombination of the *Z/AP* reporter may or may not reflect activation of the *Rosa-NotchIC* transgene, some $HPAP^+$ cells that have acquired markers of endocrine or goblet differentiation may not have expressed *NotchIC*. Second, NotchIC expression may not impose an absolute block to secretory differentiation, but may merely bias cells toward an enterocyte fate. Because Paneth cells could not be quantitated by using this approach, we cannot determine whether Notch inhibits all secretory fates equally or has a stronger effect on goblet and enteroendocrine differentiation.

Activation of Notch in the Embryonic Intestine. The finding that Notch promotes the enterocyte lineage at the expense of secretory lineages contrasts with its activity in another endoderm-derived tissue (the pancreas) as we have previously shown that Notch activation in the embryonic mouse pancreas arrests pancreatic progenitors in a precursor state (17). We thus sought to determine the impact of Notch activation in the embryonic intestine.

To gain control of Notch activity during embryogenesis, we used an allele of *Pdx1* in which the tTA replaces the endogenous *Pdx1* coding sequence (13). The Notch pathway was activated by mating *Pdx1-tTA* mice to *tetO-NotchIC* responder mice. Because *Pdx1* is expressed in the proximal intestine (Fig. 2*D Inset*), this system permitted us to examine the effect of misexpressing NotchIC on intestinal development. Crosses yielded bigenic *Pdx1-tTA;tetO-NotchIC* embryos with no pancreas and perturbed foregut morphogenesis ($n = 36$; Fig. 2 *B* and *C*). A large cyst, in continuity with the stomach and intestine, replaced all

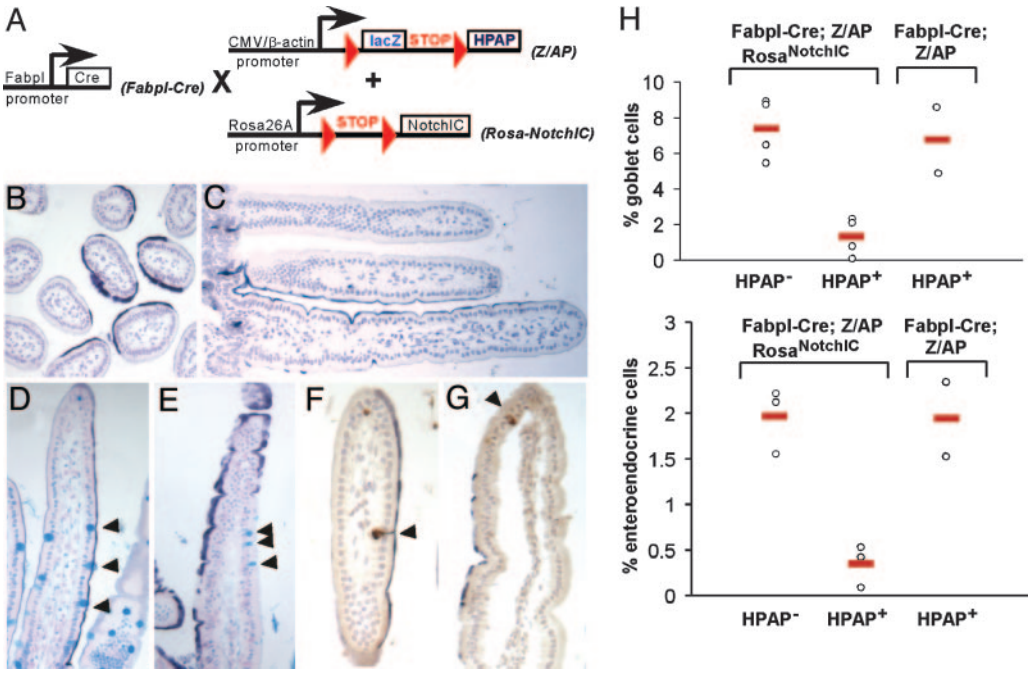

Fig. 1. Misexpression of *NotchIC* in intestinal progenitor cells. (*A*) Transgenic *Fabpl-Cre* mice were bred to *Z/AP* reporter and *Rosa-NotchIC* strains. Trigenic mice expressing Cre activate *NotchIC* in a subset of crypt progenitor cells and their progeny through the excision of a ''floxed'' transcriptional stop sequence (STOP). Transcription of the HPAP transgene is activated in these clones (from the *Z/AP* reporter allele) by the same mechanism, permitting *NotchIC*-expressing clones to be recognized by the concomitant expression of HPAP. (*B* and *C*) The intestine of an *Fabpl-Cre;Z/AP* adult mouse stained for HPAP (hematoxylin counterstain). Cre activity in intestinal progenitor cells results in HPAP activity in an arc of cells in villi that have been cut in cross section (*B*) or in a portion of two adjacent villi derived from the same crypt cut in saggital section (*C*). (*D–G*) Reduced goblet cell differentiation (*D* and *E*) and endocrine cell differentiation (*F* and *G*) in villi exposed to NotchIC. Costaining for HPAP and alcian blue shows a paucity of goblet cells (arrowheads) in HPAP-positive regions of *Fabpl-Cre;Rosa-NotchIC;Z/AP* trigenic intestines (*E*) compared with *Fabpl-Cre;Z/AP* controls (*D*). Costaining for HPAP and chromogranin A/B shows a paucity of endocrine cells (arrowheads) in HPAP-positive regions of *Fabpl-Cre;Rosa-NotchIC;Z/AP* trigenic intestines (*G*) compared *FABPCre;Z/AP* controls (*F*). (*H*) Notch signals block secretory fates in adult intestine. *Fabpl-Cre;Rosa-NotchIC;Z/AP* and *Fabpl-Cre;Z/AP* mice were double stained for HPAP and alcian blue (*Upper*) or chromogranin A/B (*Lower*). Cells in $HPAP^+$ and $HPAP^-$ villus segments were scored separately according to genotype by two independent observers. Each circle indicates data from a single mouse, presented as a percentage of total cells counted. The mean percentage is indicated with a red line. Additional details are listed in the Table 1.

intervening structures (Fig. 2 *D* and *E*); the cystic epithelium expressed markers of pancreatic progenitor cells (Fig. 5, which is published as supporting information on the PNAS web site), as described (25).

We focused on the region distal to the foregut cyst, an area corresponding to the duodenum. *In situ* hybridization of E12.5 intestine with a NotchIC riboprobe demonstrated the highest levels of endogenous Notch1 transcripts in a ring of intestinal mesenchyme (Fig. 2*F Upper*), as described (7). In *Pdx1-tTA; tetO-NotchIC* bigenic embryos, NotchIC transcripts were found in the epithelium of the cyst and the duodenum (Figs. 2*F* and 5), although duodenal expression became progressively restricted during development (Fig. 3*G*). Activation of the Notch pathway in this region was confirmed by staining for Pdx1 to visualize the E12.5 duodenum (Fig. 2*G*) and then staining adjacent sections for the Notch target Hes1. In wild type, Hes1 expression was detected in the primitive pancreatic epithelium and the duodenal mesenchyme, but not the duodenal epithelium. By contrast, the bigenic duodenum exhibited epithelial Hes1 staining (Fig. 2*H*), indicating Notch pathway activation in the intestinal epithelium.

Despite a normal gross appearance (bracketed area in Fig. 2*C*), the villi of bigenic animals were reduced in number and were blunted and misshapen (Fig. 3*A*). Litters exposed to tetracycline throughout pregnancy were phenotypically normal, indicating complete repression of the transgene (data not shown). Given the role of Notch in other developmental systems, we hypothesized that Notch might interfere with the differentiation of stem cells and examined the intervillus segments, which constitute the transient amplifying compartment of the intestine (26). BrdUrd staining was markedly decreased in the intervillus compartment of bigenic embryos compared with wild type (Fig. 3*B*). To assess the status of Wnt signaling, we examined the expression of *conductin/axin2*, a Wnt-regulated gene whose expression reflects active Wnt signaling (27). No difference in *conductin/axin2* expression was detected by *in situ* hybridization (Fig. 3*C*).

Several mechanisms could account for the loss of the proliferative layer and villus dropout in bigenic embryos. Notch signals might (*i*) inhibit the differentiation of stem cells into transient amplifying cells, (*ii*) inhibit the proliferation of the transient amplifying population, (*iii*) cause their precocious differentiation into a mature intestinal cell type, or (*iv*) kill cells within the proliferative compartment by a direct toxic effect of the *NotchIC* transgene. TUNEL assays performed at two developmental stages did not demonstrate an increase in apoptosis relative to wild-type embryos (Fig. 3 *D* and *E*). However, the percentage of $Pdx1^+$ cells in bigenic embryos had declined significantly by E15.5, shortly after the onset of villus morphogenesis (compare Figs. 2*G* and 3*F*). In parallel, loss of transgene expression was observed in bigenic embryos, because expression of *NotchIC* within the duodenum was limited to a small number of cells by the end of gestation (Fig. 3*G*). Notably, the villi that formed in bigenic embryos contained all intestinal cell types (enterocytes, enteroendocrine cells, and goblet cells) despite the underlying architectural abnormalities (Fig. 6, which is published as supporting information on the PNAS web site).

These results suggest that Notch blocks stem/progenitor cell differentiation or inhibits the proliferation of the transient

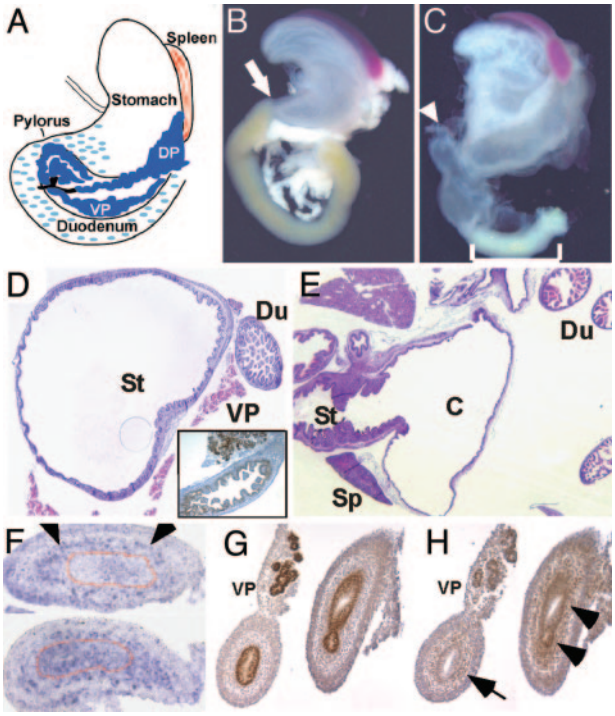

Fig. 2. Conditional misexpression of NotchIC in the embryonic intestine. (*A*) Drawing of the embryonic foregut and midgut structures analyzed in this study. The intestinal expression domain of Pdx1 is indicated by blue dots, with the dorsal and ventral pancreas shaded solid blue. (*B* and *C*) Whole mount photographs of E18.5 wild type (*B*) and *Pdx1-tTA;tetO-NotchIC* bigenic embryos (*C*), showing loss of pancreatic tissue and defective morphogenesis. The wild-type pylorus (arrow) is obliterated in the bigenic (arrowhead). The small intestine distal to the cyst (*C*, bracket) appears grossly normal. (*D* and *E*) Hematoxylin/eosin (H&E) staining of the peri-pyloric structures in E18.5 wild type (*D*) and bigenic embryos (*E*). At this stage, Pdx1 is widely expressed in the pancreas and duodenum (*D Inset*). In the bigenic embryo, the distal stomach, pancreas, and proximal duodenum have been replaced by a thin-walled cystic epithelium (*E*). (*F*) *In situ* hybridization of duodenum from E12.5 wild-type (*Upper*) and *Pdx1-tTA;tetO-NotchIC* (*Lower*) embryos with a NotchIC antisense probe. Endogenous Notch1 transcripts are found predominantly in the mesenchyme of wild-type intestine (arrowheads), whereas transcripts reflecting transgene expression are also found in the epithelium of *Pdx1-tTA;tetO-NotchIC* duodenum. (*G* and *H*) Pdx1 and Hes1 immunostaining (E12.5). Adjacent sections from a wild-type (*Left*) or *Pdx1-tTA;tetO-NotchIC* bigenic embryo (*Right*) were stained for Pdx1 (*G*) or Hes1 (*H*). Pdx1 staining is observed in the branching epithelium of the wild-type pancreas and throughout the duodenal epithelium of both wild-type and bigenic duodenum (*G*). Hes1 expression in wild-type embryos is observed in the pancreatic epithelium and the duodenal mesenchyme (arrow), but only rarely in the duodenal epithelium. By contrast, Hes1 is expressed throughout the duodenal epithelium of bigenic embryos (arrowheads), indicating Notch pathway activation (*H*). C, cyst; DP, dorsal pancreas; VP, ventral pancreas; St, stomach; Du, duodenum; Sp, spleen.

amplifying population. Because Pdx1 expression is significantly reduced in the intestines of older bigenic embryos, the phenotype most likely reflects a selection for cells that do not express the transgene (by virtue of being Pdx1$^-$) and are therefore able to support normal villus development. Even though cells that express the transgene constitute a progressively smaller percentage of the total, these cells would be unable to contribute to villi, accounting for the altered morphogenesis of *Pdx1-tTA;tetO-NotchIC* embryos.

Recovery Following *NotchIC* Repression. Another way to distinguish whether the phenotypes observed in bigenic embryos result from an inhibitory activity of Notch (rather than precocious differentiation or cell death) is that the former mechanism should be

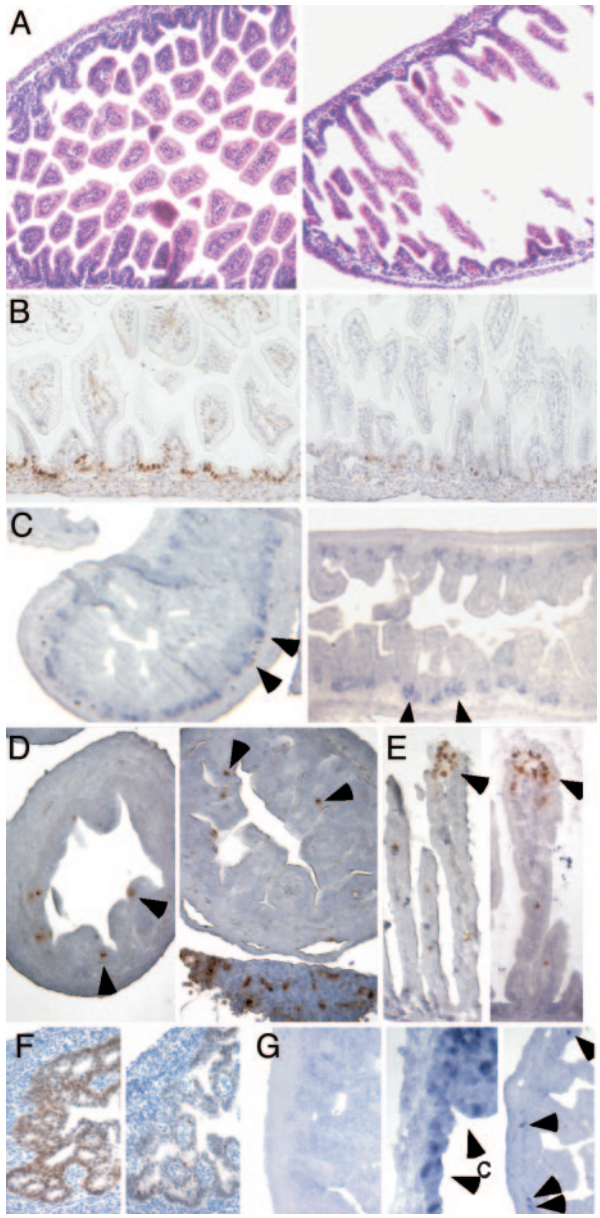

Fig. 3. Constitutive Notch signaling perturbs intestinal morphogenesis; wild-type (*Left*) and bigenic *Pdx1-tTA;tetO-NotchIC* (*Right*) embryos are compared. (*A*) H&E staining of E18.5 duodenum (in a region distal to the cyst). Compared with wild type, villi from bigenic embryos are blunted and malformed. (*B*) Disruption of the basal intestinal proliferative layer (E17.5). Pregnant mice were injected with BrdUrd 2 h before killing. Bigenic embryos exhibit a reduced number of BrdUrd$^+$ cells compared with wild-type controls. (*C*) Normal *conductin/axin2* expression assessed by *in situ* hybridization (E18.5). Despite exhibiting villus malformation, *conductin/axin2* is expressed in the duodenal crypts of bigenic embryos (arrowheads) with a distribution and level that were comparable with that of wild-type controls. (*D* and *E*) TUNEL assays were performed on E15.5 (*D*) and E18.5 (*E*) littermates. (*D*) At E15.5, rare TUNEL$^+$ cells (arrowheads) are observed in both wild-type and bigenic embryos; frequent TUNEL$^+$ cells in the adjacent liver (*Lower Right*) served as an internal control. (*E*) At E18.5, TUNEL$^+$ cells are observed at the same frequency in both wild-type and bigenic intestines, predominantly at villus tips (arrowheads). (*F* and *G*) Loss of Pdx1 and *NotchIC* transgene expression in the bigenic intestinal epithelium. At E15.5, most duodenal epithelial cells in wild-type mice express Pdx1 (left). By contrast, most cells in the duodenal epithelium of bigenic embryos have lost Pdx1 expression (right). At E18.5, the *NotchIC* transgene is expressed throughout the cyst epithelium of bigenic embryos (*G Center* arrowheads) but is detectable in only a few isolated cells in the intestine (*G Right*). NotchIC expression is below the level of detection in wild-type intestine by *in situ* hybridization at this stage (*G Left*).

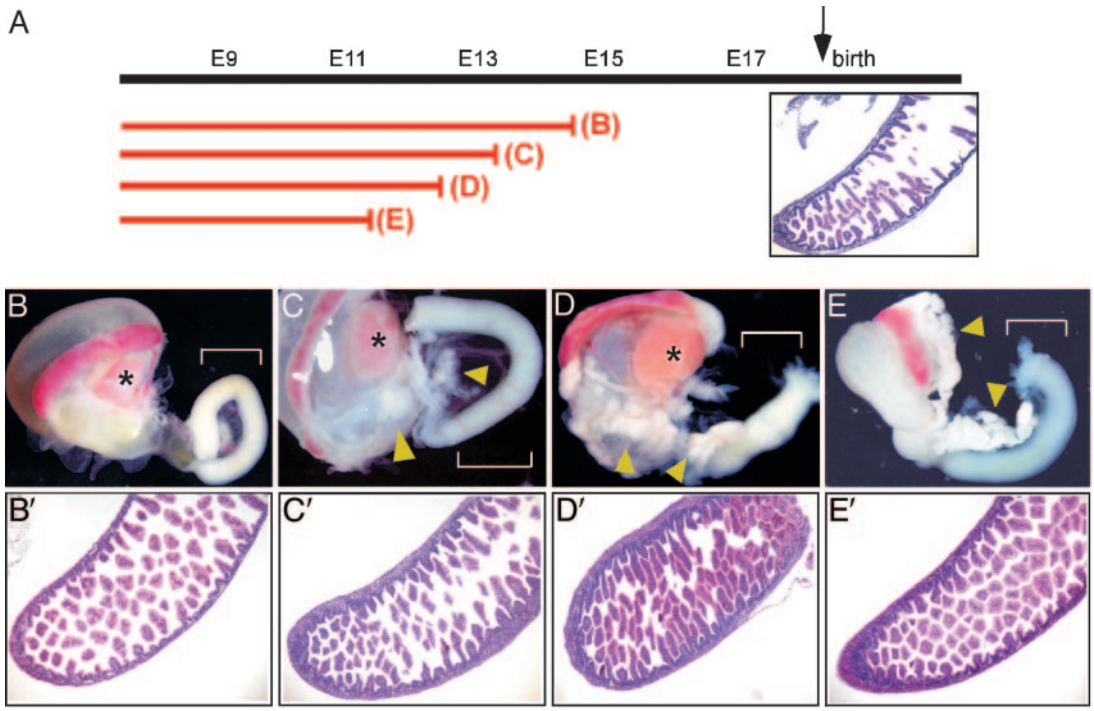

Fig. 4. The intestinal effects of Notch activation are reversible. (*A*) Scheme for temporal regulation of the *NotchIC* transgene. Each red bar indicates the time window during which the transgene was expressed (i.e., no tetracycline), corresponding to panels *B–E*. Silencing of the transgene was achieved by the addition of tetracycline (vertical bar). Embryos were killed at E18.5 (arrow) and examined grossly and histologically. A section of bigenic intestine stained with H&E is included for reference. (*B–E*) Whole mount and H&E staining of E18.5 intestines (bracketed areas) from embryos in which NotchIC has been repressed starting at E14.5 ($n = 2$; *B* and *B′*), E13.5 ($n = 4$; *C* and *C′*), E12.5 ($n = 2$; *D* and *D′*), or E11.5 ($n = 3$; *E* and *E′*). Recovery of pancreatic development (yellow arrowheads) occurs with transgene silencing at E13.5 or earlier (*C–E*), whereas recovery of intestinal morphogenesis is observed at all stages (*B′–E′*). In some dissections, a portion of the liver was left *in situ* (*).

reversible. We hypothesized that, if *NotchIC* blocks differentiation, then removal of the Notch signal might allow the differentiation of arrested stem/progenitor cells. To test this hypothesis, we silenced expression of NotchIC by adding tetracycline during midgestation (Fig. 4*A*). Repression of the transgene at various time points resulted in the progressive recovery of normal intestinal architecture and pancreas development when embryos were examined at E18.5 (Figs. 4 *B–E* and 6), demonstrating that the bigenic epithelium retains the capacity to differentiate and reconstitute normal intestinal morphology following release from a Notch signal.

Notch inhibits the differentiation of many types of progenitor cells during development, but it is unknown whether silencing of a preexisting Notch signal renders a cell capable of differentiating, or whether other signals are needed. Our results are consistent with the interpretation that Notch inhibits the differentiation of intestinal stem cells, and that release from the Notch signal is sufficient to allow differentiation to ensue. Nevertheless, we cannot rule out the possibility that Notch exerts an inhibitory activity on the proliferation of transient amplifying cells rather than the differentiation of stem cells. The effect of activating Notch within this compartment could be addressed through the use of the villin promoter, which is expressed at all stages of villus maturation (28).

A decrease in villus number and loss of the proliferative compartment is also observed in mice with defective Wnt signaling (2, 3). These similarities raise the question of whether the Notch and Wnt pathways make up a coordinated circuit that regulates intestinal growth and differentiation. Although the intestines of bigenic newborns express normal levels of the Wnt-regulated gene *conductin/axin2*, this result does not exclude the possibility that these two signaling pathways interact during intestinal development. Notch might antagonize Wnt signaling through a mechanism that is independent of this marker, or Notch could be negatively regulated by Wnt signals. Such an arrangement has precedent, because the Wnt pathway has been shown to play a role in hematopoietic stem cell renewal, possibly by regulating Notch (29). There are additional similarities between the cytodifferentiation phenotypes in the Wnt loss-of-function and our Notch gain-of-function models; specifically, both models exhibit secretory differentiation defects. Inhibition of intestinal Wnt signaling with Dkk1 causes the loss of all secretory cell types (3), whereas loss of the Wnt mediator Tcf4 is associated with defective enteroendocrine cell differentiation but preserved goblet cell differentiation (2), and thus development of the secretory lineages may be subject to more complex levels of regulation.

How do these results fit into a model of intestinal development? A paucity of progenitor cell markers prevents us from determining the relative positions of $Pdx1^+$ and $Fabpl^+$ cells in a lineage hierarchy of intestinal differentiation. Nevertheless, it is likely that $Pdx1^+$ cells are precursors of at least a subset of multipotent intestinal progenitor cells in postnatal life. *NotchIC* misexpression therefore leads to two quite different phenotypes depending on the time (and cell type) in which it is expressed. In the embryonic gut, NotchIC seems to perturb development by arresting the differentiation (or expansion) of stem/progenitor cells whereas, in the adult, NotchIC inhibits secretory fates without altering the enterocyte lineage. It is well established that the response to Notch signals depends critically on the cellular context in which it is received (5). In that respect, our model for how Notch signals regulate intestinal development resembles the mechanism used by other tissues that are maintained throughout life by stem cells: the blood and skin. In adult blood, Notch

promotes hematopoietic stem cell self-renewal whereas, in committed progenitors, Notch plays a central role in lymphoid vs. myeloid and T vs. B lymphocyte cell fate decisions (6). Similarly, in the skin, Notch inhibits the differentiation of precursor cells but also regulates the epidermal vs. hair cell fate decision (30). These similarities suggest that, in adult homeostasis, Notch may serve a more general function as a dual-activity gatekeeper, maintaining stem cells in an undifferentiated state, and biasing fate outcome during differentiation of committed progenitors.

Note Added in Proof. During the preparation of this manuscript, three other relevant reports were published (31–33). These studies support our conclusion that Notch regulates the secretory-absorptive cell fate decision in intestinal development.

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