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.

**T**issues 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, alters intestinal lineage commitment (2, 3). Wnt signals are integrated with signals from bone morphogenetic 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 tet-O-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 tet-O-NotchIC stud males. The other mouse strains used have been described: Pdx1-tTA (13), Fabpl (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...
were hybridized overnight at 50°C–65°C; probe detection was performed by using an alkaline phosphatase-conjugated antidigoxigenin 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 MgCl2) for 15 min at 50°C. Suitable slides were then subjected to immunohistochemistry for chromogranin A/B or staining with 1% alcin 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 kit (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 expression of transgenes in intestinal progenitor cells (4) permits us to examine the effect of misexpressing the Notch1 receptor (NotchIC) by crossing Fabpl-Cre;Z/AP mice to Rosa-NotchIC transgenic mice; the Rosa-NotchIC transgene 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).

**Activation of Notch in the Embryonic Intestine.** The finding that Notch promotes the enteroendocrine cell type at the expense of the secretory fates (24) suggests that the Notch pathway may have a role in the development of the gut endoderm. In the human placenta, HPAP stains secretory cells but not goblet cells (23). In the newborn mouse gut, HPAP stains goblet cells in HPAP villi of all ages (22). In this study, we used three different lines of transgenic mice to examine the effect of misexpressing the Notch1 receptor (NotchIC) in the mouse embryo.

**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 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
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. 2F, 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. 2F and 5), although duodenal expression became progressively restricted during development (Fig. 3G). Activation of the Notch pathway in this region was confirmed by staining for Pdx1 to visualize the E12.5 duodenum (Fig. 2G) 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. 2H), indicating Notch pathway activation in the intestinal mesenchyme.

Despite a normal gross appearance (bracketed area in Fig. 2C), the villi of bigenic animals were reduced in number and were blunted and misshapen (Fig. 3A). 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. 3B). 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. 3C).

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. 3D 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. 2G and 3F). 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. 3G). 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 stem/progenitor cells, which constitutes the transient amplifying compartment of the intestine. The process by which Notch inhibits intestinal cell differentiation and proliferation is currently under investigation.

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**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 sagittal 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 segments in villi exposed to NotchIC (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.
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

![Diagram](image-url)
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. 4A). 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 conducin/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 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 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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Table 1. Quantitation of goblet and endocrine cells in *Fabpl-Cre* mice

<table>
<thead>
<tr>
<th>Notch</th>
<th>HPAP positive:</th>
<th>HPAP negative:</th>
<th>Cre; Z/AP HPAP positive:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># counted</td>
<td>% goblet</td>
<td># counted</td>
</tr>
<tr>
<td>1</td>
<td>243</td>
<td>0.83 (0.07)</td>
<td>1129</td>
</tr>
<tr>
<td>2</td>
<td>905</td>
<td>0.05 (0.07)</td>
<td>1874</td>
</tr>
<tr>
<td>3</td>
<td>614</td>
<td>2.35 (0.42)</td>
<td>4193</td>
</tr>
<tr>
<td>4</td>
<td>3887</td>
<td>2.19 (0.32)</td>
<td>2053</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td><strong>1.36 (1.11)</strong></td>
<td><strong>7.39 (1.68)</strong></td>
</tr>
</tbody>
</table>

Intestines from mice with the indicated genotype were stained for HPAP and either alcian blue (goblet cell stain) or chromogranin A/B (endocrine cell stain). Goblet and endocrine cells within HPAP positive and negative areas were scored by two independent blinded observers. For each mouse, the mean number of cells counted is shown along with the mean frequency of goblet or endocrine cells as a percentage of the total cells counted. Standard deviations (parentheses listed next to each measurement) reflect inter-observer variation. The overall means and standard deviations for each experimental group (bold) are also shown (*P* values determined by two-sample equal variance *t* test).
Supplementary Figure 1
Supplementary Figure 2