STOMACH STEM CELLS NEED NOTCH
Select Clones Drive T-ALL Relapse
Ripk3 in T Cell Necroptosis
Self-renewing epithelia in the stomach and intestine share common features, including stem cell activity. Transit-amplifying progeny of these stem cells replicate briskly and differentiate in the small bowel into enterocytes and secretory cells, and in the glandular stomach into four principal daughter lineages: foveolar (pit), oxyntic (parietal), zymogenic (chief), and enteroendocrine (EE) cells. The stomach body or corpus is organized in monoclonal gland units that contain a luminal surface pit (foveolus), leading to a narrow isthmus, a short neck, and a wide base (see also Fig. 2 A). \[^{1}\text{H}]\text{thymidine autoradiography and ultrastructural features place gastric stem cells in the isthmus (Hattori and Fujita, 1976; Lee et al., 1982). From this location, pit cells migrate toward the lumen, whereas zymogenic, EE and most parietal cells migrate toward the base (Karam and Leblond, 1993). In the distal stomach, or antral-pyloric segment, glands are short and carry rare chief or parietal cells but abundant mucous and EE cells (Lee and Leblond, 1985). Stem cells in this part of the stomach lie close to the gland base, and like their counterparts in the intestine, the crypt base columnar cells (Barker et al., 2007), they express the cell surface marker Lgr5 (Barker et al., 2010). Wnt–β-catenin signaling, in particular, is essential for proliferation of intestinal crypt cells (van der Flier and Clevers, 2009), but its functional requirement in most gastric stem cells is unclear.

The intestinal epithelium also responds to the Notch signaling pathway, inactivation of which arrests cell replication and induces secretory cell metaplasia (van Es et al., 2005). Unregulated Notch signaling in the fetal intestine enhanced cell proliferation and inhibited goblet and EE cell differentiation in one study (Fre et al., 2005) and caused reversible progenitor loss and villus dysmorphogenesis in another (Stanger et al., 2005). Furthermore, Notch and Wnt signaling seem to cooperate in intestinal tumorigenesis (Fre et al., 2009; Rodilla et al., 2009). Targeted disruption of Hes1, a presumptive target of Notch signaling (Iso et al., 2003), increases the number of EE cells in the stomach and intestine (Jensen et al., 2000). Recent evidence suggests that Notch regulates progenitor proliferation and secretory cell differentiation through a single
intestine-restricted basic helix–loop–helix transcription factor, Math1 (Kazanjian et al., 2010; van Es et al., 2010; Kim and Shviddasani, 2011). Because Math1 is not expressed in the stomach (Yang et al., 2001), Hes1 functions in stomach EE cell differentiation must occur through a Notch– or Math1– independent pathway (Jensen et al., 2000). It is also unknown if the Notch pathway affects gastric corpus progenitors, which are not strictly Wnt dependent, or promotes gastric tumorigenesis as it does in cooperation with Wnt in the intestine (Fre et al., 2009; Rodilla et al., 2009).

Here we report that Notch signaling begins early in stomach epithelial development and is largely confined to the proliferative zone in the isthmus of adult glands. Inhibition of Notch function impairs proliferation and has a modest effect on cell lineage allocation. Remarkably, Notch activation in lineage-committed stomach epithelial cells converts them into progenitors that produce all daughter cell types. Over time, proliferation of these dedifferentiated progenitors accelerates, leading to formation of dysplastic adenomas that show focal activation of Wnt signaling. In contrast, Notch activation by the same means in Lgr5+ antral-pyloric stem cells preserves the normal pattern of cell proliferation. Collectively, these results reveal a requirement for Notch signaling in epithelial cell proliferation throughout the digestive tract and new, context-dependent roles for Notch in gastric stem cell homeostasis and tumorigenesis.

RESULTS

Inhibition of Notch signaling in embryonic mouse stomach exposes a competitive disadvantage

We examined expression of all Notch receptors, Notch1–4, ligands (Jagged1 and 2 and Delta-like 1, 3, and 4), and its best characterized target gene, Hes1 (Ohtsuka et al., 1999), during mouse gut development, using RNA in situ hybridization at embryonic days (E) 12, 14, 15, and 17. Multiple ligands and receptors are expressed in fetal gastric epithelium, especially the Notch1 receptor and Jagged2 and Delta3 ligands (Fig. 1 A; Fig. S1; and not depicted); subepithelial signals were proportionately weaker. Consistent with expression of the signaling components, we readily detected Hes1 mRNA and protein in embryonic stomach epithelium (Fig. 1, A and B).

To examine the requirement for Notch signaling in stomach development, we first tested a conditional loss-of-function mutation in mice. As multiple ligands and receptors could be functionally redundant, we focused on the obligate Notch effector RBP-Jκ, also known as CSL (Kopan and Irlan, 2009). Cre recombinase–dependent disruption of RBP-Jκ is an established tool to interrogate Notch function (Han et al., 2002). To express Cre in fetal stomach, we used Ore1-Cre transgenic mice, which transiently express Cre in the gut endoderm around E11 (Grieshammer et al., 2008). Crosses between Ore1-Cre and Rosa26YFP reporter mice (Srinivas et al., 2001) confirmed mosaic expression throughout the E15 digestive tract, and subsequent crosses yielded Ore1-Cre;RBP-Jκ<sup>fl/fl</sup>;Rosa26YFP progeny in Mendelian ratios. RBP-Jκ<sup>fl/fl</sup> mutant and RBP-Jκ<sup>−/−</sup> controls showed a mean of 67 and 72% YFP+ gut endodermal cells at E15, respectively (Fig. 1 C), reflecting mosaic Cre-mediated recombination in their precursors.

Consistent with the growth stimulatory effect attributed to Notch in intestinal progenitors (van Es et al., 2005) and known effects of crypt competition, YFP+ villi declined by 70% in 6-wk-old Ore1-Cre;RBP-Jκ<sup>fl/fl</sup>;Rosa26YFP intestines compared with Ore1-Cre;RBP-Jκ<sup>−/−</sup>;Rosa26YFP controls (Fig. 1 C). Similarly, YFP+ gastric gland units were reduced by 47% compared with control animals (Fig. 1 C). Thus, RBP-Jκ–null intestinal and stomach epithelia both face a significant competitive disadvantage, allowing rapid dominance by clonal units that are proficient in Notch signaling.

In adult mouse intestine, conditional RBP-Jκ inactivation or Notch inhibition by γ-secretase inhibitors arrests crypt cell proliferation and induces goblet cell metaplasia (van Es et al., 2005). As neither of these defects was evident in YFP+ villi of adult Ore1-Cre;RBP-Jκ<sup>fl/fl</sup>;Rosa26YFP intestines (Fig. 1 D), we considered the possibility that transient Cre expression in Ore1-Cre mice caused inefficient recombination at the RBP-Jκ locus, allowing residual Notch activity in both the YFP− (as a result of no recombination [mosaicism]) and YFP+ (as a result of recombination at the ROSA26YFP locus and monoallelic recombination at one RBP-Jκ<sup>fl</sup> locus) daughter cells. To test this possibility, we isolated YFP+ cells using flow cytometry and genotyped RBP-Jκ alleles using PCR (Laky and Fowlkes, 2007). Indeed, adult YFP+ cells showed evidence for both deleted and nondeleted floxed sequences, confirming incomplete recombination (Fig. 1 E). Nevertheless, significantly reduced representation of YFP+ cells in the adult Ore1-Cre;RBP-Jκ<sup>fl/fl</sup>;Rosa26YFP gut (Fig. 1 C) provides genetic evidence of a requirement for Notch signaling in maintenance of self-renewing stem cells.

Notch pathway inhibition in adult stomach arrests epithelial proliferation

Hes1 expression in the adult mouse intestine is restricted to proliferating crypt progenitors (Schröder and Gossler, 2002; van Es et al., 2005). Likewise, in adult stomach, Hes1 antibody (Ab) stained cells in the isthmus of corpus (Fig. 2 B) and antral (Fig. 2 C) glands, and most but not all Hes1+ cells coexpress the proliferation marker Ki67. These results suggest that Notch also signals in proliferating adult stomach cells. To inactivate Notch signaling in wild-type adult mice, we used the known Notch antagonist dibenzazepine (DBZ; Milano et al., 2004; Riccio et al., 2008). As others have reported (van Es et al., 2005), administration of 20 µmol/kg for 5 d arrested cell replication and induced severe goblet cell metaplasia in increased numbers of Alcian blue–avid mucous cells at the isthmus of gastric glands (Fig. S2 C), indicating pathway-specific effects. Although stomach epithelial proliferation was only mildly reduced at the end of the 5-d treatment, proliferation in corpus and antral glands was virtually abolished 2 d later, leaving few Ki67+ cells in some isthmi and no Ki67+ cells in most glands (Fig. 2, D and E). Notch inhibition also resulted in increased numbers of Alcian blue–avid mucous cells at the
base of antral glands (Fig. 2 F) and a small increase in corpus chromogranin A+ EE cells (Fig. S2 D). 5 d of DBZ exposure led to death by day 8, precluding assessment of delayed effects. These results demonstrate a role for Notch signaling in gastric epithelial proliferation, parallel to that in Wnt–dependent intestinal crypts.

**Figure 1. Expression of Notch signal components and their role in development of gastric gland units.** (A) In situ hybridization for Notch1, Jagged2, and the Notch target gene Hes1 in E15 and E17 mouse stomach epithelium. Boxed areas are shown at higher magnification in the insets. Additional data appear in Fig. S1. (B) Hes1 immunostaining of E16 mouse stomach. (C) Clonal YFP+ gastric gland units and intestinal villi in Osr1-Cre;RBP-Jk$^{fl/fl}$;Rosa26$^{YFP}$ (MUT) mice compared with Osr1-Cre;RBP-Jk$^{+/fl}$;Rosa26$^{YFP}$ (CONT) controls. Counts at E15 represent endodermal cells; at P7 and P42, the counts represent villi (intestine, Int) or glands (stomach, St). Representative examples of stomach and intestine YFP staining are shown from E15 mouse embryos and 42-d-old adults. Dotted lines demarcate the mesenchyme-epithelium boundary; trace mesenchymal signal is a blood vessel artifact. (D) Staining of adult (6 wk) Osr1-Cre;RBP-Jk$^{fl/fl}$;Rosa26$^{YFP}$ intestine with trefoil factor 3 (TFF3, left, co-stain) and Ki67 (right two panels, serial sections) Ab. (E) Flow sorting of YFP$^-$ (gate P5) and YFP$^+$ (gate P4) cells, followed by PCR genotyping for recombination at the RBP-Jk locus (right). Preservation of LoxP-flanked RBP-Jk sequences in YFP$^+$ cells indicates inefficient, likely monoallelic Cre-mediated deletion. The deleted and nondeleted RBP-Jk alleles give 700- and 500-bp PCR products, respectively (Laky and Fowlkes, 2007). GAPDH provides a positive control. Bars, 50 µm. Quantitation in C was made using three embryos or mice per group; all other experiments were repeated twice with similar results.

**Activation of Notch signaling in parietal cells confers stem cell properties**

The restriction of Notch pathway activity to progenitor cells (Fig. 2, B and C) prompted us to test the consequences of ectopic Notch signaling in differentiated cells, using transgenic mice that express Cre recombinase under control of
the parietal cell–specific $H^+/K^+$-ATPase β-subunit (Atp4b) promoter (Syder et al., 2004). To delineate the domain of Cre expression, we crossed Atp4b-Cre and Rosa26[YFP] mice. YFP signal appeared in all Atp4b-expressing cells (Fig. 3 A and Fig. S3 A), confirming the reported lineage-restricted pattern (Syder et al., 2004). A few basally located YFP+ cells failed to stain with Atp4b Ab, but expressed the chief cell–specific product, gastric intrinsic factor (Gif, Fig. 3 C); however, most Gif+ cells did not express YFP and, as expected, Atp4b and Gif staining never overlapped (Fig. S3 B). These results suggest that Cre might express in bipotential progenitors of parietal and chief cells. To examine this possibility, we treated mice with the S-phase label 5-ethynyl-2′-deoxyuridine (EdU). In the isthmus of rare glands, we observed YFP-positive cells that lacked Atp4b but had incorporated EdU, indicating recent cell replication (Fig. 3, A and B, arrows). As expected, the proliferation marker EdU and the Atp4b marker of terminal parietal cytodifferentiation did not overlap.

Figure 2. Role of Notch in proliferation of adult gastric epithelial progenitors. (A) Diagram of a glandular unit in adult gastric corpus. Stem cells in the isthmus give rise to four major progeny: pit, chief, parietal, and EE cells. (B) Hes1 and Ki67 expression in the isthmus of gland units in the adult gastric corpus. Sequential tissue sections reveal coexpression of Hes1 and proliferation marker Ki67 (arrows). Hes1 luminal surface staining is nonspecific. Boxed areas are shown at higher magnification in the insets. (C) Coexpression of Hes1 and Ki67 in the isthmus of antral glands, where both markers are expressed in cells closer to the base than in the corpus. (D) Adult gastric epithelial Ki67 expression 5 and 7 d after administration of 20 μmol/kg DBZ in 0.5% METHOCEL E4M or 0.5% METHOCEL E4M only (Mock). (E) Quantitation of proliferating cells in stomach glands after DBZ exposure. Bars represent mean ± SD. (F) Alcian blue stain reveals antral mucous cells. Bars, 50 μm. Analysis in E was done using three mice per group. Experiments in F used three mice, with staining done in duplicate; all other experiments were repeated twice, with similar results. Corp, corpus; Ant, antrum; Int, intestine.
To induce Notch activity in this differentiated compartment, we crossed Atp4b-Cre mice with the Rosa26NICD knock-in strain; expression of the Notch1 intracellular domain (NICD1) in these mice requires excision of an upstream Lox-STOP-Lox cassette (Murtaugh et al., 2003). To trace lineages, we retained the Cre-dependent Rosa26YFP allele (Fig. 3 D), expecting Cre to both activate the Notch pathway and mark all descendant cells with YFP. The resulting constitutive presence of NICD1 profoundly altered the pattern of YFP staining in gastric glands. In Atp4b-Cre;Rosa26NICD/YFP (NICD) gastric glands, most gastric corpus units (mean 92 ± 0.88%) expressed YFP throughout the gland (Fig. 3, E, G, and I), indicating derivation of all resident cells from NICD1-expressing parietal cells. Two possibilities may explain this result: (a) parietal cells with Notch activation outcompete other lineages and occupy whole glands, or (b) parietal cells with Notch activation dedifferentiate into multipotential progenitors or stem cells, giving rise to all lineages. To distinguish between these possibilities, we stained tissues for the chief cell–specific marker Gif, the EE cell product chromogranin A, the parietal cell marker Atp4b, and the foveolar cell–specific dye periodic acid Schiff. YFP signals, which are confined to parietal cells in control mice, were now readily evident in all four epithelial cell types (Fig. 4, A–C, and Fig. S3 F), indicating derivation of all resident cells from NICD1-expressing parietal cells. Two possibilities may explain this result: (a) parietal cells with Notch activation outcompete other lineages and occupy whole glands, or (b) parietal cells with Notch activation dedifferentiate into multipotential progenitors or stem cells, giving rise to all lineages. To distinguish between these possibilities, we stained tissues for the chief cell–specific marker Gif, the EE cell product chromogranin A, the parietal cell marker Atp4b, and the foveolar cell–specific dye periodic acid Schiff. YFP signals, which are confined to parietal cells in control mice, were now readily evident in all four epithelial cell types (Fig. 4, A–C, and Fig. S3 F), indicating that they derive from YFP+ progenitors. Notch pathway activation in rare parietal cells present in the antrum also triggered conversion into multipotential cells that reconstituted entire glands (Fig. S3 G). Although the NICD1 transgene contains an IRES-GFP cassette, the low level of nuclear GFP was undetectable by fluorescence.
microscopy and did not interfere with robust detection of YFP in the transgenic mice. Nevertheless, to exclude possible fluorescence artifacts in Rosa26YFP mice, we crossed the Rosa26loxP allele (Soriano, 1999) into the Atp4bCre;Rosa26NICD background and also observed β-galactosidase staining throughout gland units (Fig. S3 H). GFP immunostaining further confirmed that whole glands were derived from NICD1-expressing cells (unpublished data).

Constitutive Notch activity thus converts cells that are committed to the parietal or a mixed parietal-chief cell lineage into stem or multipotential progenitors that give rise to all stomach epithelial cell types. Glands derived from NICD1-expressing progenitors appeared functionally intact, with all cell types present in normal proportions and location, except for a reduced fraction of EE cells, consistent with the known effect of Notch signaling in inhibiting EE cell differentiation in the intestine. As Atp4b gene expression normally initiates by E17, NICD1-induced dedifferentiation could in principle reflect cellular reprogramming during development; in this case, stem-like activity should be apparent at or soon after birth. However, 8- and 15-d-old stomachs showed a mosaic of YFP+ and YFP− glands, with a progressive increase in the YFP+ fraction (Fig. 4 D), and it took 3–6 wk for the vast majority of corpus units to become uniformly labeled. These results demonstrate a gradual conversion of glands in postnatal life.

NICD1-induced dedifferentiation could occur either in mature parietal cells or, if the Atp4b transgene is activated earlier, in their precursors. Several observations support the latter possibility. First, YFP or LacZ expression in 6–8% of glands remained confined to parietal cells (Fig. 3 G and Fig. S3 H), which indicates that mature NICD1-expressing

Figure 4. Notch-mediated conversion of parietal cell precursors to multipotent progenitors. (A) Co-localization of YFP with Ab staining for the chief cell-specific marker intrinsic factor (IF). Yellow and blue lines point to the base of three consecutive gastric glands that show IF Ab and YFP signals in the same cells. The gland pointed in blue is shown at high magnification in each inset. (B) Co-localization of YFP with Ab staining for the EE cell-specific marker chromogranin A (ChgA). The yellow arrow points to a typical cell expressing both YFP and ChgA; the white arrow shows ChgA expression in a cell derived from one of the few glands that escape NICD-mediated conversion. (C) Two sequential tissue sections demonstrate broad expression of YFP in foveolar (pit) cells, which stain specifically with periodic acid Schiff (PAS). Boxed areas in the two consecutive images are magnified in the two panels to the far right. (D) Highly mosaic YFP expression in 8- and 15-d-old Atp4b-Cre;Rosa26GFP mice, with total gland involvement by 6 wk. 8-d-old Atp4b-Cre;Rosa26−/YFP CONT stomach is shown in the left image. (E) Nonoverlapping patterns of EdU+ proliferating cells (red) and terminally differentiated Atp4b+ cells (green) in Atp4b-Cre;Rosa26NICD/YFP stomachs. Dashed lines delineate the luminal edge of stomach glands. Bars, 50 µm. All experiments were performed on at least three mice and tissues were stained in duplicate, with similar results.
cells can avoid conversion. Second, rare Gfp+ chief cells and EdU-incorporating cells coexpressed YFP (Fig. 3, A–C, arrows), hinting at some Cre-expression within proliferative bipotential precursors. Third, parietal cells greatly outnumber their precursors. Had this number of mature cells converted into progenitors, both gland architecture and cell proportions would be markedly distorted, which was not the case. Indeed, the rate at which whole glands expressed YFP in the first weeks of life (Fig. 4 D) approximates the rate of parietal cell turnover (Karam and Leblond, 1993). Fourth, if mature parietal cells were to dedifferentiate, then it should be possible to capture the progenitor property of cell replication in some cells expressing the terminal marker Atp4b. We therefore analyzed EdU localization in relation to Atp4b expression in mice treated with EdU at different ages. As we observed in wild-type mice (Fig. S3 C), Atp4b and EdU did not localize in the same cells in Atp4b-Cre;Rosa26NICD/YFP mice (Fig. 4 E). Collectively, these results suggest that a parietal or bipotential precursor is the more likely target of Notch-induced conversion into multipotent progenitors than are fully mature parietal cells.

Notch activity did not seem to interfere with cell matura-
tion or epithelial function, and whole glands remained YFP+ at 18–20 wk, suggesting long-term reconstitution. However, ongoing Atp4b-Cre transgene activity precludes making a distinc-
tion between continual replenishment of multipotential progenitors and stable conversion into stem cells. As whole glands never expressed YFP in the absence of NICD (Fig. 3, F and H), the important point is that NICD converts a cell that is substantially committed toward lineage differentiation into one that can generate all epithelial cell types.

**Notch-induced dedifferentiation leads to formation of adenomas that show focal Wnt pathway activation**

Hes1 is overexpressed in intestinal adenomas in APCMin mice (van Es et al., 2005), Notch activation increases intestinal adenomas in APCMin;1638N mice, and Jagged1 haploinsufficiency in APCMin mice reduces polyp size (Fre et al., 2009; Rodilla et al., 2009). As these observations all point to a role for Notch signaling in intestinal tumorigenesis, we followed the consequences of constitutive Notch activity in the stomach epithelium longitudinally. Between 4 and 8 wk of age, the dramatic effect of NICD1 on stomach cell dedifferentiation was accompanied by overtly normal patterns of cell proliferation (Fig. 5 A). By 18 wk, however, the zone of cell replication in Atp4b-Cre;Rosa26NICD/YFP stomachs was significantly expanded, well beyond the isthmus (Fig. 5 B), and multiple sessile adenomas became evident. Within these adenomas, proliferation was further increased, replicating cells were distributed haphazardly, and cell nuclei were enlarged and dys-
plastic (Fig. 5, C–E; Fig. S4; and not depicted). Thus, cells that dedifferentiate as a result of Notch activation proliferate excessively and generate dysplastic adenomas, indicating that Notch activation unmasks a tumorigenic potential latent within parietal cells or their immediate precursors. Inciden-
tially, some foci of dysplasia within adenomas showed loss of the mature cell markers Atp4b and Muc5AC, which were robustly expressed in neighboring tissue but not in these dys-
plastic foci (Fig. 5, F and G).

Because patients with familial adenomatous polyposis and mice with targeted disruption of the Apc tumor suppressor gene develop stomach polyps in addition to intestinal lesions (Offerhaus et al., 1999; Tomita et al., 2007), we examined the gastric adenomas in Atp4b-Cre;Rosa26NICD mice for evidence of Wnt pathway activation, starting with nuclear localization of the canonical Wnt pathway effector β-catenin (Clevers, 2006). We did not detect nuclear β-catenin in the stomachs of <30-wk-old mice, but focal patches of intense activity appeared in mice by ~1 yr, comparable to the signal in intestinal adenomas in ApcMin mice (Fig. 5 H). In some areas, this signal was confined to the base of stomach corpus glands (Fig. 5 J); in other areas it distributed evenly within adenomas (Fig. 5 H). To verify Wnt pathway activation with other, more sensitive molecular markers, we isolated stomach epithelial cells and used quantitative RT-PCR to measure expression of transcripts that are known to respond to Wnt signaling in antral stomach and intestinal adenomas (Van der Flier et al., 2007; Barker et al., 2010). 5 of the 10 Wnt target genes we assessed in this fashion showed significantly increased levels in Atp4b-Cre;Rosa26NICD stomach epithelium compared with control littermates (Fig. 5 I). Immunohistochemical examination of two such overexpressed markers, CD44 and Sox9, revealed broader distribution than nuclear β-catenin, but the latter signal always coincided with the other markers (Fig. 5 J). Thus, over time the adenomas that form within NICD-active stomach epithelium show evidence for focal activation of the canonical Wnt signaling pathway.

**Adenoma formation upon prolonged Notch activation depends on cellular context**

Recently identified stomach stem cells resemble their intesti-
nal counterparts in expressing the surface marker Lgr5, but are restricted to the antral-pyloric segment and a small area of the corpus near the squamo-columnar junction (Fig. 6 A); Wnt activation in Lgr5+ intestinal or stomach antral stem cells induces adenomas (Barker et al., 2009, 2010). To determine if the aberrant cell proliferation induced by Notch activation in dedifferentiated parietal cells would occur in a native stem cell population, we introduced the Rosa26NICD and Rosa26LacZ alleles into Lgr5-CreERT2 mice (Fig. 6 B). Notch-activated Lgr5+ progeny contributed to whole glands in the antrum (Fig. 6 C) and esophageal junction (Fig. 6 D), reflecting known stem cell activity. Expanded cell replication and focal adenomas were always apparent in Atp4b-Cre;Rosa26NICD mice by 18 wk (Fig. 5 B). In contrast, β-galactosidase–stained gastric glands showed the same, normal pattern of cell proliferation as their neighbors, with no proliferating cells lying outside the isthmus and few if any parietal cells within β-galactosidase+ glands (Fig. 6, C and D). A trivial explanation for the lack of enhanced cell replication could be that Lgr5-Cre is inefficient in excising the Lox-STOP-Lox cassette at the Rosa26NICD allele, so that LacZ+ glands may not coexpress NICD. To exclude this possibility, we examined stained tissues for GFP, which is
expressed as a nucleus-localized protein from an internal ribosome entry site in the NICD construct (Fig. 6 B). Although nuclear GFP was expressed throughout recombined antral glands, the Ki67 proliferation marker was restricted to the typical basal zone of cell replication (Fig. 6 E). Accelerated proliferation and adenomas are thus highly context-dependent in the stomach, occurring uniformly in dedifferentiated corpus stem cells but not in resident Lgr5+ antral stem cells.

**DISCUSSION**

In the digestive epithelium, Notch signaling has been implicated in homeostasis and cell lineage allocation in Wnt-dependent intestinal crypts but its role in the closely related mucosal lining of the stomach is less well understood. Our results reveal Notch signaling both in the developing stomach endoderm and in the isthmus of adult gastric glands, coincident in the latter with an isthmal progenitor population. Blockade of Notch activity impairs epithelial proliferation, whereas constitutive Notch activation in committed parietal or bipotential parietal-chief cell precursors has the remarkable effect of gradually converting them into multipotential progenitors. These progenitors initially proliferate normally and give rise to all gastric epithelial cell types. As mice age, gland units derived from NICD1-expressing parietal cells show excessive cell replication and produce adenomas with areas of poor differentiation and foci of Wnt signaling pathway activation. Because Notch activation in resident Lgr5+ gastric antral stem cells, in contrast, does not disturb proliferation or produce adenomas, we conclude that dedifferentiation of a committed cell provides a distinctive niche that is especially sensitive to unregulated Notch signaling.

Signaling mechanisms that regulate stomach epithelial renewal play an important part in the response to injury and in gastric cancer, but are largely unknown. Using pharmacologic inhibition, we demonstrate that, like their intestinal counterparts, gastric stem and progenitor cells also depend on Notch signaling. A slower effect on stomach cell proliferation compared with the intestine might reflect different turnover rates or distinct downstream mechanisms in the two organs. Intestinal cell cycle arrest and secretory cell metaplasia are believed to reflect activation of the transcription factor Math1 (Kazanjian et al., 2010; van Es et al., 2010; Kim and Shivdasani, 2011), which is absent from the stomach (Yang et al., 2001). Thus, stomach Notch signaling probably operates through an alternative factor, which affects cell proliferation less quickly and secretory cells less profoundly than Math1 does in the intestine.

**Figure 5.** Adenoma formation with focal Wnt pathway activation in progenitors dedifferentiated as a result of unregulated Notch activity. (A and B) The zone of Ki67 staining in Atp4b-Cre;Rosa26<sup>NCf5fp</sup> stomach epithelium at 6 wk (A) and 18 wk (B). (C-E) Hematoxylin and eosin (H&E) staining reveals focal adenomas in 18-wk-old Atp4b-Cre;Rosa26<sup>NCf5fp</sup> stomach (C and D), including subregions of dysplasia with enlarged nuclei and stratified cells. The boxed area in C is shown at higher magnification in D. (E) Gland structure in littermate controls. (F and G) Impaired cell maturation in scattered foci within Notch-induced adenomas. Nuclear β-catenin staining, indicating activation of the canonical Wnt signaling pathway, in 52-wk-old mutant mouse stomachs (bottom right; inset shows nuclear localization at high magnification). Also shown are intestinal adenomas in APC<sup>Min+</sup> mice (top left), control littermates (CONT, top right), and younger NICD mutants, e.g., 30 wk (bottom left). (H) Quantitative RT-PCR analysis of Wnt target genes on 1-yr-old Atp4b-Cre;Rosa26<sup>NCf5fp</sup> and littermate control stomach epithelial cells. Results are normalized with respect to Gapdh mRNA and expressed as the mean ± SD. Total epithelial cells isolated from three mice per genotype were analyzed in duplicate, with similar results. (I) Immunohistochemistry for CD44, Sox9, and β-catenin in control and NICD-expressing mouse stomach. Boxed areas are magnified in the insets. Bars, 50 µm. All tissue analyses used four mice and were done in duplicate, with similar results.
Our most unexpected and unusual finding is that a single in vivo manipulation of parietal cells reprograms cells with restricted potential into those that can produce all stomach epithelial cell lineages. In contrast, lone Notch activation in the intestine alters crypt proliferation subtly and requires a functional Wnt pathway (Fre et al., 2009). This difference might reflect the fact that we activated Notch in committed gastric cells, whereas others used Villin-Cre transgenic mice to activate Notch throughout the intestinal epithelium, including native stem cells (Fre et al., 2009). Our results suggest that parietal cell precursors are the likely targets of unregulated Notch activity, but continuous expression of the Atp4b-Cre transgene precludes establishing whether they dedifferentiated into bona fide stem cells or multipotent progenitors. Tetracycline control of Cre recombinase activity (Gossen and Bujard, 1992) might, in the future, allow testing of long-term reconstitution to distinguish between these possibilities. A second key question pertains to the mechanisms that act downstream of Notch signaling to induce dedifferentiation. Canonical Wnt signaling controls homeostasis in intestinal crypts and perhaps also in the gastric antrum but not in most of the gastric corpus (Barker et al., 2010). As Wnt signaling is activated only very late and focally after corpus epithelial dedifferentiation, it is not likely to mediate the Notch effect. Four or fewer defined transcription factors can reprogram mature embryonic and adult cells into pluripotent stem cells (Takahashi and Yamanaka, 2006), and it will be interesting to determine if the same factors control the form of reprogramming we report in the stomach in vivo.

Overexpression of Notch receptors 1 and 3 is statistically significant in both the intestinal and diffuse subtypes of human gastric cancer (Chen et al., 2003), hinting at a role for Notch activity in some cases. Furthermore, dedifferentiated progenitors in aged Atp4b-Cre;Rosa26CRE2 mice proliferate excessively and eventually develop adenomas, perhaps much as induced pluripotent stem cells are prone to form tumors in mice (Okita et al., 2007). Adenomas in the abnormal gastric epithelium showed small areas of intense nuclear β-catenin signal and increased expression of several Wnt target genes. Among these genes, CD44 was recently proposed as a marker of gastric cancer stem cells (Takaishi et al., 2009). Thus, Notch-mediated dedifferentiation might, over time, increase the pool of potential tumor-initiating cells. Wnt signaling may play a part in human and murine tumors of the gastric antrum (Offerhaus et al., 1999; Tomita et al., 2007), but probably has little role in corpus cancers; the significance of focal Wnt pathway activity in Notch-induced gastric adenomas is uncertain. In our study, Notch activation in gastric antral stem cells did not affect cell proliferation or produce adenomas. Because dedifferentiated progenitors in NICD-expressing gastric epithelium generate all resident cell types and the cell of adenoma origin is therefore unclear, our results suggest the importance of antecedent dedifferentiation in tumor formation. Collectively, our observations reveal a requirement for Notch signaling in gastric epithelial homeostasis, affecting the proper balance between self-renewal and differentiation that is essential to avoid tumors.

MATERIALS AND METHODS

Experimental animals. RBP-JκCreERT2 mice (Han et al., 2002) were provided by S. Artaudus-Takonas (Harvard Medical School, Boston, MA) with permission from T. Honjo (Kyoto University, Kyoto, Japan). Osr1-Cre mice (Grieshammer et al., 2008) were provided by G. Martin (University of California, San Francisco, CA). Atp4b-Cre mice (Syder et al., 2004) were provided by J. Gordon (Washington University, St. Louis, MO). Rosa26VP (Srinivas et al., 2001), Rosa26CreERT2 (Soriano, 1999), Rosa26CreERT2-YFP (Murthaugh et al., 2003), and Lgr5CreERT2;Rosa26Cre mice (Barker et al., 2007) mice were purchased from The Jackson Laboratory. Lgr5CreERT2;Rosa26Cre and littermate control mice were treated with 2 mg tamoxifen (Invitrogen) by i.p. injection for 5 consecutive days. Animals were housed under specific pathogen–free conditions and handled in accordance with protocols approved and monitored by the Dana-Farber Cancer Institute Animal Care and Use Committee.

DBZ. The γ-secretase inhibitor DBZ was synthesized at Syncom (Groningen, Netherlands) in 0.5% (wt/vol) hydroxypropylmethylcellulose (METHOCHEL E4M; Dow Chemicals) and 0.1% (wt/vol) Tween-80 in water, and injected i.p. (20 µmol/kg) on 5 consecutive days.

In situ hybridization. Tissues were fixed overnight in 4% paraformaldehyde at 4°C, embedded in OCT compound (Sakura), and sectioned into 5-µm sections. Antigen was retrieved in 10 mM Na citrate buffer (pH 6.0) and endogenous peroxidase activity blocked in methanol and 3% H2O2. Frozen tissues were treated with 1 µg/ml Proteinase K (Roche) followed by 10 µM triethanolamine in 0.2% acetic anhydride. Samples were hybridized overnight with digoxigenin-labeled antisense probes at 60°C. DNA templates for riboprobes were gifts from A. McMahon (Harvard University, Boston, MA). Slides were washed in 2X SSC, incubated with alkaline phosphatase-conjugated digoxigenin Ab (1:2,000), and treated with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

Immunohistochemistry and histology. Tissues were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, embedded in paraaffin, and cut into 5-µm sections. 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 and sectioned at 5-µm thickness. After blocking with 5% fetal bovine serum, samples were incubated with the following Ab: Muc5ac (45M1; 1:500; Novocastra), chromogranin A (1:500; Abcam), Muc4b (1:1,000; Thermofisher Scientific), GIF (1:200,000; gift from D. Alpers, Washington University, St. Louis, MO), Hes1 (1:1,000; gift from N. Brown, University of Cincinnati, OH), K67 (MM1, 1:1,000; Vector Laboratories), PCNA (1:300; Neomarkers), CD44 (1:500; Biogenesis), β-catenin (1:500; BD), Sox9 (1:300; Millipore), and TFF3 (1:300; gift from D. Podolsky, University of Texas Southwestern, Dallas, TX). For immunofluorescence, cryosectioned samples were incubated with Texas red–conjugated anti-mouse or anti-rabbit IgG (1:300; Jackson ImmunoResearch Laboratories) and analyzed using a microscope (Eclipse E800; Nikon). Paraffin-embedded samples were incubated with biotin-conjugated anti-mouse or anti-rabbit IgG (1:300; Vector Laboratories), and color reactions were developed with VECTASTAIN avidin-biotin complex ABC kit (Vector Laboratories) and diaminobenzidine substrate (Sigma–Aldrich). Hes1 staining was amplified with Tyramide Signal Amplification kit (PerkinElmer). Samples were examined with a compound microscope (BX41, Olympus). Paraffin-embedded samples were dehydrated, stained in Alcan blue solution (pH 2.5), and counterstained with nuclear fast red. EdU staining was performed using the Click-iT Edu Alexa Fluor 594 Imaging kit (Invitrogen), following the manufacturer’s instructions. We used fluorescence microscopy to count 250 endodermal cells (YFP+ and YFP−) at E15 and 200 gastric corpus glands and distal intestinal villi in 7- and 42-day-old Osr1-Cre;RBP-JκCreERT2; Rosa26VP mice (n = 3 for each stage). K67+ cells were counted in 100 vertically sectioned gastric glands from 3 mice for each DBZ treatment condition. Means and SDs were calculated using Microsoft Excel.

LacZ staining. Dissected tissues were frozen immediately in OCT, sectioned at 16 µm thickness, and stained with β-galactosidase kit (Active Motif) according to the manufacturer’s protocol.
Quantitative PCR analyses of mouse stomach epithelial cells. Adult mouse stomachs were washed with PBS and incubated in PBS containing 1×IM EDTA at 37°C for 30 min. Epithelial cells were collected and their RNA was harvested using TRIzol reagent (Invitrogen) and reverse transcribed using SuperScript enzyme. cDNA was tested by SYBR green master mix (Applied Biosystems) and qPCR using gene-specific primers (Van der Flier et al., 2007; Barker et al., 2010; Spandidos et al., 2010), as follows: Lgr5, forward 5'-CCTACTCGAAGATCTCCACGCT-3' and reverse 5'-GATTGCGTGCCAGGTCTG-3'; SP5, forward 5'-TTGGGCTTCTCCAGCCTTTG-3' and reverse 5'-ATGCTTCCAGTGATAGCCTTC-3'; CDH2, forward 5'-GATCGCCACTCCAGCTACTTC-3' and reverse 5'-CTGCCTCAGACTCAACTC-3'; Cdx2, forward 5'-GATGCCACATCCTGACATTC-3' and reverse 5'-CCGAAATCGAGGGAGA-3'; Sox9, forward 5'-GAAGGGCTTGGAAGGGGA-3' and reverse 5'-GCTTGCACGGTGTCGGGTGC-3'; Vgfa, forward 5'-CCTGCGCGGAGAACTCAGG-3' and reverse 5'-CCCTCTCTGTGATAGGGACAC-3'; and reverse 5'-CCTACTCGAAGATCTCCACGCT-3' and reverse 5'-GATTGCGTGCCAGGTCTG-3'.

Online supplemental material. Fig. S1 shows broad expression of Notch signaling components in E14 mouse embryos. Fig. S2 demonstrates efficient transgene expression and localizes active Notch signaling to the gastric epithelium at 18 wk. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101737/DC1.

REFERENCES

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