Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells

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SUMMARY
Notch signaling is known to regulate the proliferation and differentiation of intestinal stem and progenitor cells; however, direct cellular targets and specific functions of Notch signals had not been identified. We show here in mice that Notch directly targets the crypt base columnar (CBC) stem cell to maintain stem cell activity. Notch inhibition induced rapid CBC cell loss, with reduced proliferation, apoptotic cell death and reduced efficiency of organoid initiation. Furthermore, expression of the CBC stem cell-specific marker Olfm4 was directly dependent on Notch signaling, with transcription activated through RBP-Jκ binding sites in the promoter. Notch inhibition also led to precocious differentiation of epithelial progenitors into secretory cell types, including large numbers of cells that expressed both Paneth and goblet cell markers. Analysis of Notch function in Atoh1-deficient intestine demonstrated that the cellular changes were dependent on Atoh1, whereas Notch regulation of Olfm4 gene expression was Atoh1 independent. Our findings suggest that Notch targets distinct progenitor cell populations to maintain adult intestinal stem cells and to regulate cell fate choice to control epithelial cell homeostasis.

KEY WORDS: Olfactomedin 4, Lgr5, Atoh1, Gamma-secretase inhibitor, Tuft cell, Mouse

INTRODUCTION
The intestinal epithelium is continuously replenished from stem and progenitor cell populations located in the crypts. Recent studies have identified an actively dividing stem cell, termed the crypt base columnar (CBC) stem cell, as a self-renewing progenitor responsible for maintaining the epithelium (Barker et al., 2007). Although the CBC stem cell was originally described many decades ago based on its unique cellular morphology (Cheng and Leblond, 1974b; Cheng and Leblond, 1974a), the more recent identification of CBC stem cell markers, including Lgr5, Ascl2 and Olfm4 (Barker et al., 2007; van der Flier et al., 2009), has greatly facilitated the study of this stem cell population. Replicating CBC stem cells can self-renew or give rise to rapidly dividing transit-amplifying (TA) cells, which are short-lived progenitors that differentiate into mature cell types, including absorptive enterocytes, hormone-secreting enteroendocrine cells, mucus-secreting goblet cells, antimicrobial peptide-secreting Paneth cells and chemoattracting tuft cells (Barker et al., 2007; Gerbe et al., 2011). The factors regulating stem cell self-renewal versus differentiation are not well understood, although competition for limited niche binding sites has been proposed to control total CBC stem cell number (Snippert et al., 2010).

The role of Notch signaling in the regulation of both progenitor cell proliferation and cellular differentiation in the intestine is well established; Notch signaling promotes differentiation to the absorptive cell lineage rather than to the secretory cell lineage (Jensen et al., 2000; Fre et al., 2005; Stanger et al., 2005; van Es et al., 2005; Riccio et al., 2008; Gerbe et al., 2011; Pellegrinet et al., 2011). Notch pathway inhibition of the transcription factor atonal homolog 1 (Atoh1) provides the crucial mechanism regulating cell fate choice, and Atoh1 expression appears to be both required (Yang et al., 2001; Shroyer et al., 2007) and sufficient (VanDussen and Samuelson, 2010) for the program of secretory cell differentiation. In general, disruption of Notch signaling results in increased Atoh1 expression and loss of proliferation coupled with secretory cell hyperplasia, whereas hyperactive Notch signaling results in decreased Atoh1 expression and in expansion of the proliferative zone with increased numbers of absorptive enterocytes. Accordingly, genetic depletion of Notch pathway components, including the crucial Notch DNA-binding protein RBP-Jκ (Rbpj – Mouse Genome Informatics) (van Es et al., 2005), both Notch1 and Notch2 receptors (Riccio et al., 2008) or both delta-like (Dll) 1 and 4 ligands (Pellegrinet et al., 2011), results in decreased cellular proliferation in the intestinal crypts together with secretory cell hyperplasia. Similar phenotypes have been observed in rodents after treatment with γ-secretase inhibitors (GSIs) (Milano et al., 2004; Wong et al., 2004; van Es et al., 2005), which block an essential protein cleavage event in the activation of Notch signaling, or with a combination of neutralizing antibodies specific for the Notch1 and Notch2 receptors (Wu et al., 2010). Conversely, activation of constitutive Notch signaling in the mouse intestinal epithelium expands the proliferative zone and represses secretory cell differentiation (Fre et al., 2005; Stanger et al., 2005).

Notch is likely to target distinct stem and progenitor cell populations to regulate different aspects of intestinal homeostasis, although specific cellular targets had not been definitively identified. Crucial components of the Notch signaling pathway, including the Notch1 and Notch2 receptors, the ligands jagged 1, Dll1 and Dll4, and the Notch target genes hairy and enhancer of split 1 (Hes1), Hes5 and Hes6, have been localized to the proliferative zone of the
intestinal crypts (Jensen et al., 2000; Schroder and Gossler, 2002; Benedito and Duarte, 2005; Crosnier et al., 2005; van Es et al., 2005; Riccio et al., 2008). Importantly, lineage tracing from cells undergoing active Notch signaling identified long-lived progenitors that gave rise to all the mature epithelial cell types (Vooijs et al., 2007; Pellegrinet et al., 2011), suggesting that Notch signaling was active in a stem cell. More specifically, Notch regulation of the CBC stem cell was suggested by the enrichment of Notch1 receptor mRNA in this cell type (van der Flier et al., 2009).

Although these studies build a strong case for the idea that the Notch pathway is active in adult intestinal stem cells, the significance of this signaling pathway for stem cell function is unknown. In this study, we demonstrate that Notch signaling in CBC stem cells is required for stem cell proliferation and survival. Furthermore, we demonstrate that Notch regulation of the CBC stem cell is Atoh1 independent, whereas Notch regulation of epithelial cell fate is Atoh1 dependent, suggesting that Notch targets distinct aspects of progenitor cell function to regulate intestinal epithelial cell homeostasis.

MATERIALS AND METHODS

Mice

C57BL/6 mice were used unless otherwise noted. Atoh1lacZ/lacZ (Yang et al., 2001) (gift from N. F. Shroyer), Lgr5-EGFP-IRE-S-creERT2 (Lgr5-GFP) (Barker et al., 2007) (Jackson Laboratories, #008875), Vili-Cre (Pinto et al., 1999) and Rosa26Notch1 (Murtaugh et al., 2003) (Jackson Laboratories, #008159) mice were genotyped by PCR as recommended by the Jackson Laboratory or with the primers listed in supplementary material Table S1. Mice were maintained specific pathogen-free under a 12-hour light cycle and with the primers listed in supplementary material Table S1. Mice were maintained specific pathogen-free under a 12-hour light cycle and protocols were approved by the University of Michigan Committee on the Use and Care of Animals.

Notch inhibition

Administration of dibenzazepine (DBZ; SYNCOM, Groningen, The Netherlands) was as described (van Es et al., 2005). Mice aged 2 months were injected intraperitoneally (i.p.) (30 μmol/kg DBZ or vehicle daily) for 5 days and fasted overnight before tissue collection on day 6.

A mixture of neutralizing antibodies directed against the Notch1 and Notch2 receptors was administered by i.p. injection to BALB/c mice at 5 μmol/kg DBZ or vehicle daily for 5 days and fasted overnight before tissue collection on day 6. Notch inhibition

Fetal intestine organ culture

The morning of the vaginal plug was considered embryonic day (E) 0.5. For organ culture, E15.5 intestine segments (A–D, proximal to distal) were cultured at the air–liquid interface on Transwell plates (Costar 3428) in BGI/b media (Gibco) with 0.1 mg/ml ascorbic acid (Sigma) and Pen-Strep (Gibco). GSI (DAPT; EMD4 Biosciences, Gibbstown, NJ, USA) was added at 40 μM unless otherwise indicated, and cultures were incubated for up to 4 days with daily media changes.

Bromodeoxyuridine (BrdU) administration and tissue collection

Adult mice were injected with 50 μg/kg BrdU (5 mg/ml in 0.9% saline; Sigma) 2 hours prior to tissue collection. Intestine was dissected as follows: duodenum (4 cm distal to pylorus), jejunum (4 cm at the midpoint), ileum (4 cm proximal to cecum) and colon (4 cm distal to cecum). The proximal 1 cm of each tissue segment was processed for paraffin embedding after fixation in 4% PBS-buffered paraformaldehyde at 4°C overnight. Fetal organ culture segments A and D were similarly processed for paraffin embedding. Duodenal tissue was collected from newborn Vil-Cre; Rosa26Notch1 mice and processed for RNA collection.

Histological analysis

Paraffin sections (5 μm) were stained with periodic acid–Schiff (PAS)/Alcian Blue (Newcomer Supply, Middleton, WI, USA) to assess mucin-containing goblet cells. Immunostaining was performed as described (Lopez-Diaz et al., 2006). Primary antibodies were used as described (Keeley and Samuelson, 2010; VanDussen and Samuelson, 2010) or as follows: goat anti-Cga (1:100, Santa Cruz), rabbit anti-DCAMKL1 (1:50, Abcam), chicken anti-GFP (1:700, Aves Labs), rabbit anti-Ki67 (Thermo Scientific, 1:200), rat anti-Mmp7 (1:100) (Fingleton et al., 2007) and rabbit anti-Sox9 (1:150, Millipore). For immunofluorescence, secondary antibodies (1:400; Invitrogen or Jackson Immunoresearch) conjugated to Alexa Fluo 488, Alexa Fluo 555, FITC or Cy3 were combined with DAPI nuclear stain (ProLong Gold, Invitrogen). For Ki67 and Sox9 staining, antigen retrieval was performed with Triology solution (Cell Marque, Rocklin, CA, USA). Microscopy was performed with a Nikon E800 or Olympus BX-51 equipped with a SPOT or Olympus DP70 digital camera, respectively. Confocal microscopy was performed with an Olympus FV500. For morphometric analysis of CBC stem cells, jejunal sections from Lgr5-GFP mice were co-stained for Ki67 and GFP. A total of 1673 and 1562 crypts were counted from vehicle-treated (n=4) and DBZ-treated (n=4) mice, respectively.

For electron microscopy, jejunum and ileum from three mice from each treatment group were evaluated. Small transverse sections of intestine were fixed for 2 hours in 2% glutaraldehyde and 2% formaldehyde (prepared fresh from paraformaldehyde) in PBS, postfixed for 45 minutes with 1% OsO4, dehydrated and Epon embedded. Ultrathin sections were stained with uranyl acetate and lead citrate, and at least ten images were recorded digitally for each intestine using a Philips CM-100 electron microscope.

Analysis of gene expression

RNA was isolated from the distal 2 cm of each intestinal segment using Trizol (Invitrogen), followed by DNase treatment using the RNaseasy Mini Kit (Qiagen). For intestinal organ cultures, RNA was isolated from segments B and C with the RNeasy Mini Kit. Reverse transcriptase (RT) reactions (50 μl) used 1 μg RNA and the Iscript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed as described (Jain et al., 2006) with SYBR Green dye and the primers listed in supplementary material Table S2. Expression levels were determined with triplicate assays per sample and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (Gapdh), which remained the same in all samples.

Intestinal organoid culture

Jejunal tissue (~10 cm) was incubated in PBS containing 4 mM EDTA and 1 mM DTT for 30 minutes at 4°C on a rotating platform. After removing villi by light shaking, the tissue was transferred into a tube with PBS. Crypts were released by vigorous shaking then passed through a 70-μm filter (Becton Dickenson). Crypts were quantitated, pelleted (13 g, 7 minutes) and resuspended in 50 μl Matrigel (BD Biosciences) supplemented with 50 ng/ml EGF (R&D Systems), 5 ng/ml WNT3A (R&D Systems), 1 μg/ml R-spondin 1 (R&D Systems) and 100 ng/ml noggin (PeproTech). After polymerization at 37°C for 30 minutes, 0.5 ml culture medium was added to each well and the culture was maintained as described (Gracz et al., 2010).

Twenty-four hours post-plating, organoid cultures were treated with vehicle or 25 μM DAPT daily for 5 days. Four representative organoids from each condition were digitally imaged under bright-field microscopy. Organoid area was measured using ImageJ (NIH, http://imagej.nih.gov/ij/). For analysis of organoid stem cell activity, isolated crypts were cultured in 24 wells for 24 hours before daily treatment with vehicle or 25 μM DAPT for 2-5 days, followed by measurement of organoid initiating activity after passing. Treated organoids were mechanically dissociated by passing both media and Matrigel through a 1-ml pipette tip seven times and a 30-gauge needle four times, split 1:3, and plated in Matrigel as described above with the addition of 1 μM jagged 1 (AnaSpec, San Jose, CA, USA) and without DAPT. Organoids were counted 2 days post-}

OLF46 luciferase constructs and transfection analysis

We used previously described plasmid constructs for human OLF46 (4270LM4-luciferase) (Chin et al., 2008), the Notch transcriptional inhibitor dominant-negative mastermind (dnMAML) (MC5V-dnMAML) (Maillard et al., 2004) and the Notch pathway activator 3XFlag-NICD1 (Ong et al., 2006). RBP-Jk binding sites were identified using the
consensus (C/A/T)(G/A)TG(G/A/T)GAA (Tun et al., 1994). Deletion of the human OLFM4 promoter sequence used the overlap extension PCR technique with outer primers RVprimer3 (5’-CTAGCAAAAAT-AGGGTGTCC-3’) and GLprimer2 (5’-CTTATGTTTTGGGC- GTCTTCCA-3’) (Promega, PGL-3-basic) and the inner mutant primers listed in supplementary material Table S4. Deletion of RBP-Jk sites used the QuikChange Mutagenesis Kit (Stratagene/Agilent Technologies) and the primers listed in supplementary material Table S3. All constructs were verified by DNA sequencing.

LS174T human colon cancer cells (ATCC #CL-188) were grown in MEM (Gibco) containing 10% fetal calf serum, sodium pyruvate, non-essential amino acids and Pen-Strep-Glut. Transient transfections used Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cell lysates were collected at 24 hours post-transfection and luciferase activity was assayed in triplicate with a PerkinElmer Victor luminometer using 20 μl of cell lysate and Dual Luciferase Reagents (Promega). Luciferase activity was normalized to total protein content as determined using the BCA Protein Assay Kit (Thermo Scientific).

Chromatin immunoprecipitation (ChIP)
LS174T cells were transfected with pCMV (control) or 3×Flag-NICD1 as described above. ChIP was performed according to published protocols from Upstate Biotech with minor modifications (Patel et al., 2007). Sonication was performed on ice with five 20-second pulses using a microtip probe sonicator (Branson Sonifier 250) with output control set to 2.5. Immunoprecipitation used 15 μg of chromatin and 5 μg antibodies – either rabbit IgG (Jackson ImmunoResearch) or anti-Notch1 (Bethyl Labs, Montgomery, TX, USA), which targets the C-terminus of the Notch1 receptor. The precipitated chromatin was reconstituted in water and qRT-PCR quantification of DNA relative to input was performed in triplicate with the primer sequences listed in supplementary material Table S4.

To ensure that the Notch1 antibody specifically immunoprecipitated FLAG-tagged NICD, 50 μl of supernatant was removed from the ChIP assays after incubation with protein-A beads, then 2 μl 5 M NaCl and 10 μl 6× Laemmli sample buffer were added and heated at 95°C for 5 minutes followed by crosslink reversal for 4 hours at 65°C. A sample (40 μl) was separated by SDS-PAGE and immunoblotting was performed with anti-FLAG antibody as previously described (Patel et al., 2007) (supplementary material Fig. S5).

Statistical analysis
Quantitative data are presented as mean ± s.e.m. The effect of a treatment between similar sample groups was analyzed with Student’s t-test, whereas the effects of genotype within a treatment group and the effects of various expression constructs on luciferase activity were analyzed by one-way ANOVA followed by Dunnett’s post-test; where P<0.05 was considered significant.

RESULTS
Notch signaling is required for CBC stem cell homeostasis
To determine the importance of Notch signaling for intestinal stem cells, we used the Lgr5-GFP mouse strain (Barker et al., 2007) to identify and track CBC stem cells after treatment of adult mice with the global Notch pathway inhibitor DBZ. GFP-positive cells were located at the base of the crypts and commonly observed to be dividing in vehicle-treated controls (Fig. 1A). By contrast, there were fewer GFP-positive cells in DBZ-treated crypts, and the remaining GFP-expressing cells were weakly staining and commonly misshapen (Fig. 1B). This finding was supported by decreased GFP mRNA abundance in DBZ-treated intestine (Fig. 1C). Morphometric analysis showed an 86% reduction in GFP-positive crypts (Fig. 1D) and a reduction in the number of GFP-labeled cells in each positive crypt, with an average of 4.1 cells in the controls and 2.1 cells per expressing crypt in DBZ-treated intestine (Fig. 1E). This analysis suggested that inhibition of Notch signaling led to an overall reduction in CBC stem cells.

We examined cellular proliferation and apoptotic cell death to determine potential mechanisms for reduced CBC stem cell numbers with Notch inhibition. Co-staining for GFP and Ki67 demonstrated a marked reduction in proliferating CBC stem cells in DBZ-treated intestine, with 59% of GFP-positive cells co-expressing Ki67 in vehicle (veh) or DBZ (n=4) (Fig. 1; data not shown). In addition, we detected rare apoptotic CBC cells in the DBZ-treated intestine after co-inhibition for GFP and activated caspase 3 (Fig. 1F). By contrast, caspase-positive CBC cells were not observed in vehicle-treated crypts. Reduced cellular proliferation coupled with cell death...
is consistent with the observed decrease in CBC stem cell numbers. The reduction in stem cell number predicts that Notch inhibition would diminish overall stem cell activity. This was tested in crypt organoid cultures. Treatment with the GSI DAPT showed a marked reduction in organoid growth (supplementary material Fig. S1) and subculturing after discontinuation of DAPT demonstrated that Notch inhibition reduced the efficiency of new organoid initiation, consistent with reduced stem cell activity (Fig. 1G). Together, these studies demonstrated that Notch signaling is crucial for the maintenance of intestinal CBC stem cells.

**Notch regulates Olfm4 expression**

To confirm that Notch pathway inhibition regulates CBC stem cells, we measured the expression of the marker genes Olfm4, Lgr5 and Ascl2. This revealed a striking reduction in Olfm4 mRNA abundance in DBZ-treated intestine (Fig. 2A), to an extent greater than the decrease in GFP-stained CBC cell number (Fig. 1). Although GSIs appear to principally affect Notch signaling based on the similarity of the phenotypes observed with DBZ treatment and genetic inhibition of the pathway (Milano et al., 2004; van Es et al., 2005; Riccio et al., 2008), it is known that the γ-secretase enzyme complex can cleave other proteins in addition to Notch receptors (Tolia and De Strooper, 2009). Therefore, we used pathway-specific models to confirm Notch regulation of Olfm4. Treatment of adult mice with a combination of neutralizing antibodies directed against the Notch1 and Notch2 receptors (Wu et al., 2010) resulted in a marked decrease in Olfm4 mRNA abundance (Fig. 2B), similar to the effect of DBZ treatment. Conversely, Olfm4 expression was increased 1.9-fold in the intestines of newborn Vil-Cre; Rosa<sup>NotchIC</sup> mice (Fig. 2C), which exhibit constitutive activation of Notch signaling throughout the intestinal epithelium (Fre et al., 2005; Stanger et al., 2005). Similar regulation was observed in the human colon cancer cell line LS174T, with endogenous OLFM4 mRNA abundance markedly decreased after GSI treatment and increased 1.8-fold following activation of Notch signaling by transfection of a Notch1 intracellular domain (NICD1) expression construct (Fig. 2D). Notch responsiveness of OLFM4 was further confirmed in LS174T cells by decreased expression of a luciferase promoter construct (427OLF4-luciferase) after DAPT treatment and by increased expression after Notch activation with NICD1 (Fig. 2E). Together, these data show that intestinal Olfm4 expression is Notch responsive.

Expression of the CBC stem cell markers Lgr5 and Ascl2 was also reduced after Notch inhibition, consistent with the observed decreases in CBC stem cell number (Fig. 2A,B), but these decreases were modest in comparison to Olfm4. The more modest changes could be due to alteration of other signaling pathways, such as Wnt signaling, which is known to regulate the expression of both Ascl2 and Lgr5 (van der Flier et al., 2009). In addition, because Lgr5 is also expressed in short-lived progenitor cell populations (van der Flier et al., 2009), the overall level of expression might reflect a balance of reduced numbers of CBC stem cells and increased numbers of TA progenitors. Indeed, we observed increased expression of the progenitor cell marker Prom1 (Snippert et al., 2009; Zhu et al., 2009) in both DBZ-treated and Notch1/2 antibody-treated intestine (Fig. 2A,B), which would be consistent with increased numbers of short-lived progenitors after Notch inhibition.

**Inhibition of Notch signaling disturbs intestinal epithelial cell differentiation**

Histological analysis demonstrated that the general reduction in cellular proliferation in DBZ-treated intestine (Fig. 1; supplementary material Fig. S1) was coupled with precocious differentiation of progenitors into secretory cell types (Fig. 3). It is well established that Notch signaling inhibits the differentiation of secretory cells (Fre et al., 2005; Stanger et al., 2005); however, there have been conflicting reports on the specific cellular changes induced by global Notch inhibition. Although all studies have reported goblet cell hyperplasia, the effect of blocking Notch on enteroendocrine and Paneth cells has been disputed (Milano et al., 2004; van Es et al., 2005; Riccio et
expands all secretory cell types and not only goblet cells. marker lysozyme (Fig. 3G,H), suggesting that Notch inhibition (Gerbe et al., 2011; Saqui-Salces et al., 2011) and the Paneth cell marker DCAMKL1 (Dclk1 – Mouse Genome Informatics) (Fig. 3E,F) increased numbers of cells staining for the enteroendocrine marker chromogranin A (Fig. 3C,D), the tuft cell marker DBZ-treated mice (Fig. 3A,B). We did not observe any cells with normal Paneth cell morphology (i.e. large electron-dense secretory granules, non-mucus-producing) in DBZ-treated intestine. The lysozyme immunostaining in the DBZ-treated crypts appeared to overlap with the goblet cell staining (Fig. 3); thus, we co-stained for goblet (Muc2) and Paneth (Mmp7) cell markers to determine whether these cells co-expressed markers of both secretory cell types. Whereas double-positive cells were rare in vehicle-treated intestine, they were numerous in DBZ-treated intestine, where they filled elongated crypts (Fig. 4C,D). These unusual secretory cells were most abundant in the distal small intestine of DBZ-treated mice, although they were observed throughout the intestine, including small clusters of double-positive cells at the base of the colonic crypts (Fig. 4E,F). The double-positive cells also expressed Sox9, a transcription factor that is normally highly expressed in mature Paneth cells (Fig. 4G,H) (Bastide et al., 2007; Mori-Akiyama et al., 2007). Moreover, DBZ induced a general increase in the expression of cryptdins, lysozyme and ephrins, suggesting that these unusual secretory cells have initiated the Paneth cell developmental program (supplementary material Fig. S2). The expansion of double-staining cells in DBZ-treated mice suggests that Notch signaling is required to maintain the mature Paneth cell phenotype and that loss of Notch signaling stimulated the formation and accumulation of Sox9-expressing precursors co-expressing Paneth and goblet cell markers. However, because Paneth cells are not thought to directly receive Notch signals, but rather to express Notch ligands (Sato et al., 2011) and Atoh1 (Pinto et al., 2003), this is likely to be a non-cell-autonomous role of Notch signaling. Interestingly, Muc2 expression did not colocalize with the endocrine cell marker chromogranin A (supplementary material Fig. S2), demonstrating that this phenotype did not extend to all secretory cell types.

**Notch regulation of Olfm4 expression is Atoh1 independent**

Consistent with the increase in secretory cells, we observed increased expression of the Notch-regulated transcription factor Atoh1 in DBZ-treated intestine (Fig. 5A-C). We also observed increased expression of transcription factors that function downstream of Atoh1 to orchestrate secretory cell differentiation, including Gfi1 (Shroyer et al., 2005; Bjerknes and Cheng, 2010), Spdef (Gregorieff et al., 2009; Noah et al., 2010) and Neurog3 (Jenny et al., 2002; Bjerknes and Cheng, 2006; Lopez-Diaz et al., 2006) (Fig. 5D-F). Because Atoh1 has been proposed to mediate intestinal Notch effects, we tested whether Notch regulation of Olfm4 expression was Atoh1 dependent. We used the Atoh1lacZ/lacZ null mouse (Yang et al., 2001) and a fetal intestine organ culture system for this analysis due to the perinatal lethality of this mutant. Wild-type and Atoh1 null fetal intestines were cultured, and Notch signaling was inhibited by treatment with GSI. Similar to adult intestine, we observed that Notch inhibition induced a general secretory cell hyperplasia in wild-type intestine, with increased...
staining for goblet, enteroendocrine and Paneth cells (Fig. 5G; supplementary material Fig. S3). Increased lysozyme staining was particularly interesting because Paneth cells do not normally mature until after birth (Cheng, 1974). Similar to adult intestine, these lysozyme-positive cells co-stained for goblet cell markers (not shown). Consistent with previously published studies (Kazanjian et al., 2010; van Es et al., 2010; Kim and Shivdasani, 2011), we observed that increased secretory cell differentiation following Notch inhibition was completely dependent on Atoh1; goblet cells and markers were not detected in DAPT-treated Atoh1 null intestine (Fig. 5G,H), nor were enteroendocrine or Paneth cell markers (supplementary material Fig. S4). Interestingly, Atoh1lacZ– intestine expressed intermediate levels of Atoh1 as well as its downstream effectors Gfi1, Spdef and Neurog3 (supplementary material Fig. S4).

In contrast to the effects on cell fate, Olfm4 expression was similarly downregulated in null and wild-type intestine after DAPT treatment, demonstrating that Notch regulation of this CBC stem cell marker is Atoh1 independent (Fig. 5I). Our finding differs from a previous study in adult Atoh1-deficient intestine, which reported that Olfm4 was not affected by DBZ treatment in this mutant (van Es et al., 2010). This discrepancy is likely to be explained by
Our finding that Notch is required for expression in CBC is a direct Notch target gene Olfm4 cells.

that luciferase activity of 427OLFM4-luciferase construct and further lowered the basal
not shown). Moreover, co-transfection with the canonical Notch for Notch responsiveness of this luciferase construct (Fig. 6C; data

Several RBP-J consensus sites were identified in the human and stem cells suggested that Notch signaling directly targets this gene.

occupation was not observed at a distant upstream site in the (Arnett et al., 2010) (Fig. 6E). By contrast, increased NICD

signaling directly regulates this cell. To our knowledge, this is the
first direct cellular target of Notch signaling identified in the intestine.


discussion

We demonstrate in this study that active Notch signaling is required for the maintenance of CBC stem cells in the small intestine. Notch inhibition resulted in stem cell loss, with both decreased proliferation and apoptotic cell death contributing to the overall reduction in CBC stem cell number. Notch inhibition also reduced the efficiency of organoid initiation, demonstrating a requirement of Notch for stem cell function. Consistent with stem cell loss was the observation that loss of Notch signaling was irreparable, with death occurring 3 days following a 5-day GSI treatment course (data not shown). Our demonstration that Notch signaling is essential for survival of the mouse CBC stem cells differs from recent studies in Drosophila showing increased proliferation of midgut stem cells with loss of Notch (Takashima et al., 2011),

Fig. 6. OLFM4 transcription is regulated by canonical Notch signaling. (A) The proximal promoter sequence of the human OLFM4 gene, featuring three RBP-Jk consensus binding sites, an N-box, an E-box, the TATA box (underlined), transcription start site (arrow, +1) and translation start site (underlined). The proximal promoter region that contains known

ChIP of a proximal region containing RBP-Jk sites or of a distant upstream region without RBP-Jk sites was performed in LS174T cells transfected with either vector control (CS2) or NICD1. (E) ChIP of a HES1 promoter region that contains known RBP-Jk consensus sites. ChIP data were normalized by subtracting the values of the control IgG samples from those of the Notch antibody samples and are presented as the percentage of the initial chromatin input. n=4 independent experiments, qPCR performed in triplicate; **P<0.01, ***P<0.001. (D) ChIP of the proximal region containing RBP-Jk sites or of a distant upstream region without RBP-Jk sites was performed in LS174T cells transfected with either vector control (CS2) or NICD1. (C) Luciferase activity was measured in LS174T cells that were co-transfected with the full-length human 427OLFM4-luciferase construct (WT) or mutant OLFM4 luciferase constructs (Δ427-306, Δ290-161 or Δ145-56) together with either empty vector or the NICD1 expression construct (B). Mutant constructs with targeted deletions of the RBP-Jk-a (ab) or RBP-Jk-a (aa) consensus sites were transfected with NICD1, dominant-negative mastermind (dnMAML), or both (C). Bar colors correspond to the shaded sequence regions in A that were altered in each mutant construct. Transfection data are reported as fold-change relative to untreated WT controls. n=3 experiments performed in triplicate; **P<0.01, ***P<0.001.
Notch targets the CBC stem cell

Notch signaling regulates several aspects of intestinal epithelial cell homeostasis. A summary of the major findings of this study, showing that Notch regulates multiple aspects of intestinal epithelial cell differentiation. (1) Notch signaling directly targets the CBC stem cell to activate Olfm4 transcription, maintain proliferation and promote cell survival. (2) Notch acts to promote differentiation to the absorptive lineage by repressing Atoh1 transcription. Although the specific cellular target for this function has not been identified, Notch is likely to act on a transit-amplifying progenitor cell to bias cell fate choice to absorptive rather than secretory cells. (3) Notch appears to act later, in an indirect manner, to segregate the Paneth/goblet cell lineage and/or maintain the mature Paneth cell phenotype.

Fig. 7. Notch signaling regulates several aspects of intestinal epithelial cell homeostasis. A summary of the major findings of this study, showing that Notch regulates multiple aspects of intestinal epithelial cell differentiation. (1) Notch signaling directly targets the CBC stem cell to activate Olfm4 transcription, maintain proliferation and promote cell survival. (2) Notch acts to promote differentiation to the absorptive lineage by repressing Atoh1 transcription. Although the specific cellular target for this function has not been identified, Notch is likely to act on a transit-amplifying progenitor cell to bias cell fate choice to absorptive rather than secretory cells. (3) Notch appears to act later, in an indirect manner, to segregate the Paneth/goblet cell lineage and/or maintain the mature Paneth cell phenotype.

We observed a rapid and substantial reduction of Olfm4 mRNA in both adult and fetal intestine after Notch inhibition and increased Olfm4 expression after constitutive activation of Notch signaling. We demonstrated that the mechanism of human OLFM4 gene regulation was transcriptional, with promoter activity directly regulated through NICD binding to canonical RBP-Jκ consensus binding sites. This finding confirmed that Notch directly targets the CBC stem cell and establishes Olfm4 as a sensitive transcriptional readout of Notch signaling in the intestine. In contrast to Olfm4, expression of the other CBC marker genes, Lgr5 and Asecl2, was not affected by Notch inhibition, with continued expression perhaps reflecting their regulation by Wnt signaling (van der Flier et al., 2009).

Currently, the intestinal function of Olfm4 protein is unknown; however, studies in other tissues have suggested that Olfm4 has functions associated with stem cell properties. Murine Olfm4 was originally identified in hematopoietic precursor cells (Zhang et al., 2002) and has since been characterized as a secreted extracellular matrix glycoprotein that can facilitate cell adhesion and bind to cell surface cadherins and lectins (Liu et al., 2006). It is a member of a family of olfactomedin domain-containing proteins that have been suggested to function in wide-ranging cellular activities, such as cell adhesion, cell cycle regulation and tissue patterning, and have been proposed to modulate Wnt and BMP signaling (Tomarev and Nakaya, 2009). Although studies have suggested that Olfm4 regulates proliferation and apoptosis (Zhang et al., 2004; Kobayashi et al., 2007), which are processes relevant for stem cell function, the intestinal function of Olfm4 remains obscure. A recent study of Olfm4-deficient mice showed normal intestinal histology, demonstrating that this protein is not required for intestine development (Liu et al., 2010); however, intestinal stem cell function was not critically analyzed in this study. Because other olfactomedin family members are expressed in intestine (data not shown), compensatory pathways could mask the role of Olfm4 in this mutant mouse.

Our findings suggest that Notch signaling regulates multiple aspects of intestinal epithelial cell homeostasis. In addition to loss of CBC stem cells, disruption of Notch signaling had profound effects on progenitor cell proliferation and cell fate determination, including a likely non-cell-autonomous effect on the terminal differentiation and maintenance of Paneth cells. A model that depicts these different aspects of Notch regulation in the intestine is shown in Fig. 7. We observed that Notch inhibition stimulated precocious differentiation of progenitors into cells of the secretory lineage, including goblet cells, endocrine cells, tuft cells and cells co-expressing goblet (Muc2) and Paneth (lysozyme and Mmp7) cell markers. The accumulation of cells double-positive for goblet and Paneth cell markers in the crypts of DBZ-treated mice had not been previously reported. Although this phenotype was most apparent in the distal small intestine, where the DBZ-treated crypts were filled with double-positive cells, they were also observed in other regions, including the colon and immature intestine, which do not normally contain Paneth cells.

Co-expression of goblet and Paneth cell markers is a feature of ‘intermediate cells’, a rare epithelial cell type that has been proposed to be a precursor for the goblet/Paneth cell lineages (Troughton and Trier, 1969; Garabedian et al., 1997). Interestingly, we observed that many of the double-positive cells induced by Notch inhibition also expressed Prom1 (data not shown), which has been described as a progenitor cell marker in the intestine (Snippert et al., 2009; Zhu et al., 2009). Thus, a portion of the double-positive cells could represent precursors to the goblet/Paneth lineage that have incompletely segregated. However, ultrastructural analysis did not reveal any normal Paneth cells with properly formed secretory granules in DBZ-treated intestine, suggesting that Notch signaling might act to maintain function and to suppress mucin gene expression in mature Paneth cells by an indirect mechanism. Thus, although the mechanism is unclear, our data suggest that Notch signaling plays a crucial role in the segregation of the goblet/Paneth cell lineages and in the terminal differentiation and/or maintenance of the mature Paneth cell phenotype.

Previous studies had demonstrated the central role of Notch signaling for regulating cell fate choice between absorptive and secretory lineages through control of the transcription factor Atoh1 (Yang et al., 2001; Shroyer et al., 2007; VanDussen and Samuelson, 2010). Notch signaling functions to repress Atoh1 expression and thereby guide differentiation to absorptive enterocytes. The singular function of Notch signaling in this decision appears to be Atoh1 repression because Notch is not required for progenitor cell differentiation into absorptive enterocytes when Atoh1 is removed (Kim and Shivdasani, 2011). There is some controversy as to whether Notch inhibition results in only goblet cell hyperplasia (Yang et al., 2001; Shroyer et al., 2007; VanDussen and Samuelson, 2010) or the full program of secretory cell activation (Milano et al., 2004; Kazanjian et al., 2010; Kim and Shivdasani, 2011). Our study demonstrates that inhibiting Notch signaling in both immature and adult intestine leads to a robust increase in Atoh1 expression and to the induction of all four secretory cell lineages and not just goblet
cell hyperplasia. Furthermore, similar to other recent studies (Kazanjian et al., 2010; van Es et al., 2010; Kim and Shivdasani, 2011), we show that Atoh1 expression is required for increased secretory cell differentiation, as GSI treatment was unable to induce secretory cell differentiation in Atoh1-deficient intestine. By contrast, we observed that Notch regulation of Olfm4 expression was Atoh1 independent.

In summary, we have shown that Notch signaling is required for CBC stem cell homeostasis. Blocking Notch resulted in fewer stem cells due to reduced proliferation, increased differentiation and apoptotic cell loss. Importantly, we identified the CBC stem cell-specific marker Olfm4 as a direct Notch target gene. Furthermore, we have shown that the generalized secretory cell hyperplasia induced by Notch inhibition is characterized by the loss of the mature Paneth cell morphology. A recent study suggested that Paneth cells might provide essential niche signals for the CBC stem cells, including the Wnt ligand Wnt3, the Notch ligand Dll4, EGF and TGFβ (Sato et al., 2011). Perhaps the morphological changes that we observed in Paneth cells after Notch inhibition could result in the alteration of important niche signals required for normal CBC stem cell homeostasis.

The fundamental importance of Notch for the intestine underscores the challenge of targeting this pathway for treatment of human cancer and other diseases, including Alzheimer’s disease (Pannuti et al., 2010). The acute and potentially lethal intestinal toxicity observed with global Notch disruption is a serious obstacle to overcome in the design of drugs that target this pathway. Thus, developing targeted therapies that preserve Notch function in the intestine will be an important future goal.

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Supplementary material
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References


