

Notch regulation of gastrointestinal stem cells

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Abstract

The gastrointestinal (GI) tract epithelium is continuously replenished by actively cycling stem and progenitor cells. These cell compartments are regulated to balance proliferation and stem cell renewal with differentiation into the various mature cell types to maintain tissue homeostasis. In this topical review we focus on the role of the Notch signaling pathway to regulate GI stem cell function in adult small intestine and stomach. We first present the current view of stem and progenitor cell populations in these tissues and then summarize the studies that have established the Notch pathway as a key regulator of gastric and intestinal stem cell function. Notch signaling has been shown to be a niche factor required for maintenance of GI stem cells in both tissues. In addition, Notch has been described to regulate epithelial cell differentiation. Recent studies have revealed key similarities and differences in how Notch regulates stem cell function in the stomach compared to intestine. We summarize the literature regarding Notch regulation of GI stem cell proliferation and differentiation, highlighting tissue specific functions to compare and contrast Notch in the stomach and intestine.

Introduction

Notch regulates key cellular processes such as proliferation and differentiation via communication between adjacent cells. The Notch pathway is unique in that Notch signals are transmitted between adjacent cells, such that Notch activity in one cell can induce distinct function in a neighboring cell. This process termed lateral inhibition, is used in many different contexts in developing and adult tissues to establish cell boundaries, to pattern cellular differentiation and regulate stem cell function (Koch *et al.*, 2013).

In mammals, there are 4 Notch receptors (NOTCH 1-4) and 5 Notch ligands (Delta-like [DLL] 1, 3, 4 and Jagged [JAG] 1, 2), which are all transmembrane proteins (Kopan & Ilagan, 2009). Notch signaling involves the engagement of ligand-expressing (signal-sending) and receptor-expressing (signal-receiving) cells to initiate proteolytic cleavage events to release an intracellular signaling fragment of the receptor: NICD (Notch IntraCellular Domain) (Fig. 1). In intestine, the initial cleavage event excising the extracellular receptor domain occurs via the cell surface sheddase a disintegrin and metalloproteinase 10 (ADAM10) (Tsai *et al.*, 2014); whether ADAM10 also performs this function in the stomach is unknown. Notch signals are subsequently transmitted to the nucleus after an intramembrane cleavage event orchestrated by the gamma-secretase complex to release NICD. Once in the nucleus, NICD interacts with the DNA-binding protein RBPJ, converting a transcriptional repressor complex into an activator complex via recruitment of co-activators such as Mastermind. This complex can then activate transcription of Notch target genes, such as those in the hairy and enhancer of split (Hes) family. Notch responses are highly tissue specific; currently, only limited information is known about Notch target genes and their function in GI tissues.

In both intestine (Fre *et al.*, 2011; Pellegrinet *et al.*, 2011) and stomach (Demitrack *et al.*, 2015; Gifford *et al.*, 2016), lineage tracing experiments in mouse genetic models have revealed active NOTCH receptor signaling in adult stem cells, suggesting an important function for this signaling pathway in stem cell homeostasis in both tissues. Indeed, as detailed in this review, pathway inhibition or activation disrupts proliferation and differentiation, with Notch signaling required for stem cell maintenance. Progress to understand Notch regulation of GI stem cells has primarily emerged from analysis of mouse genetic and pharmacologic models. In addition, recent utilization of intestinal and gastric organoid cultures has allowed focus on epithelial cell effects without the confounding issues related to potential Notch function in other cell types (immune, neuronal, mesenchymal, vascular, etc.). The ability to generate organoids from human GI tissue samples presents a promising model system to study Notch regulation of human stem cells.

Overexpression of Notch pathway components has been demonstrated in gastric and intestinal cancers (Reedijk *et al.*, 2008; Piazzini *et al.*, 2011; Hsu *et al.*, 2012; Bauer *et al.*, 2015), which underscores the importance of this signaling pathway to promote cell proliferation. Outside of the GI tract, Notch pathway dysregulation is associated with several human cancers and thus this pathway is a prime target of therapeutic interest (Takebe *et al.*, 2015). However, the use of pan-Notch inhibitors is associated with GI toxicity due to the importance of this pathway for GI stem cell maintenance (van Es *et al.*, 2005b). Thus it is crucial to define pathway components and mechanisms of Notch function in the gut to design strategies that target components of this pathway relevant for human disease without disrupting normal GI stem cell function.

Notch regulation of intestinal epithelial cell homeostasis

Intestinal stem cells

The small intestinal epithelium is rapidly renewed by actively cycling crypt base columnar (CBC) stem cells. Each crypt contains several CBCs positioned between the Paneth cells at the crypt base (Fig. 2A). One imaging study of green fluorescent protein (GFP)-marked CBCs suggested approximately 14 stem cells reside in each intestinal crypt (Snippert *et al.*, 2010), while another study using a functional, clonal labeling technique estimated 5-7 CBCs per crypt (Kozar *et al.*, 2013). Regardless, the field of intestinal stem cell biology has largely advanced through the use of gene expression profiling and lineage tracing techniques, which first established the R-spondin receptor and Wnt target gene *Lgr5* as a marker for the active CBC stem cell population (Fig. 2A) (Barker *et al.*, 2007). Since then, other markers of these stem cells have emerged, including *Olfm4* (van der Flier *et al.*, 2009), *Ascl2* (van der Flier *et al.*, 2009), *Sox9* (Formeister *et al.*, 2009; Furuyama *et al.*, 2011) and others (Munoz *et al.*, 2012).

In addition to the active CBC stem cell, another stem cell population identified as the “+4,” “reserve,” or “quiescent” stem cell (QSC), has been described. Markers for these QSCs include *Bmi1* (Sangiorgi & Capecchi, 2008), *mTert* (Montgomery *et al.*, 2011), *Hopx* (Takeda *et al.*, 2011) and *Lrig1* (Powell *et al.*, 2012). This stem cell population differs from CBCs in that they divide infrequently and are not normally responsible for epithelial cell maintenance, but can be activated in response to loss of LGR5⁺ CBCs to support epithelial cell homeostasis (Tian *et al.*, 2011; Yan *et al.*, 2012; Buczacki *et al.*, 2013). However it remains unclear if there is a single, dedicated population of QSCs or if multiple different cell populations are capable of occupying open ISC niche spaces left absent through loss of the active CBCs. Recent gene expression profiling of the LGR5⁺ CBC population showed that these cells also express QSC markers, further questioning whether distinct stem cell populations exist (Munoz *et al.*, 2012). Furthermore the observation that crypt cells co-

expressing endocrine and stem cell markers can de-differentiate to occupy open ISC niche spaces during CBC loss (Schonhoff *et al.*, 2004; Sei *et al.*, 2011; van Es *et al.*, 2012; Van Landeghem *et al.*, 2012; Buczacki *et al.*, 2013) suggests substantial cellular plasticity exists for cells in the crypt to allow remodeling or repair to maintain homeostasis (Mills & Sansom, 2015).

The field has relied heavily on genetic mouse models to both identify and study stem cells in the intestine. This has enabled remarkable progress over the past few years as discovery of stem cell markers has allowed genetic approaches to label and manipulate stem cell populations. However, over-reliance on marker expression to define stem cells can be a limitation as outcomes may report marker gene regulation instead of stem cell function. Moreover, engineered gene alleles do not always faithfully reflect endogenous gene expression, due to patchy expression patterns or a different protein half-life for Cre or GFP. This is perhaps best illustrated by the expression pattern of the *LGR5-EGFP-CreERT2* allele that is widely used in the field, with incomplete expression in all *LGR5*⁺ CBCs and perdurance of GFP into TA cells (Barker *et al.*, 2007). Thus researchers need to be cognizant of the specific limitations of the transgene alleles employed in their study. Moreover functional and morphological criteria in addition to marker expression should be included to support conclusions regarding specific stem cell populations.

Small intestinal CBCs give rise to transit-amplifying (TA) progenitor cells, which undergo several rounds of cell division before differentiating into the mature intestinal epithelial cell types. In intestine there are two distinct categories of differentiated cell lineages: absorptive (enterocytes) and secretory (goblet, enteroendocrine, Paneth and tuft cells) (Fig. 2A). Several studies have demonstrated that the Notch pathway is key to directing progenitor cell differentiation to absorptive versus secretory cell fate (see review by (Noah & Shroyer, 2013)).

Notch regulation of ISCs

Notch receptor and ligand mRNAs have been detected in both epithelial and mesenchymal cells of the developing and adult rodent intestine (Schroder & Gossler, 2002; Sander & Powell, 2004; Shimizu *et al.*, 2014), and functional studies have confirmed active Notch signaling in ISCs. Analysis of mouse strains with Notch receptor-CreERT fusion genes demonstrated active NOTCH1 and NOTCH2 receptor signaling in CBCs by development of long-lived lineage tracing events after tamoxifen treatment (Fre *et al.*, 2011; Pellegrinet *et al.*, 2011). In addition, gene expression studies showed Notch target gene expression in intestinal crypts, with localization of *Hes1* and *Olfm4* to CBC cells (Fre *et al.*, 2011; Pellegrinet *et al.*, 2011; VanDussen *et al.*, 2012). Finally, gene expression profiling showed that Paneth cells, which are directly adjacent to CBC stem cells at the crypt base, express the Notch ligand *Dll4* (Sato *et al.*, 2011). The finding that Paneth cells express genes known to support ISC function (*Dll4*, *Wnt3*, *EGF*) suggests that these cells might function as ISC niche cells (Sato *et al.*, 2011). However, this idea is controversial because other studies have shown that Paneth cells are not required for intestinal epithelial cell homeostasis (Garabedian *et al.*, 1997; Shroyer *et al.*, 2007; Durand *et al.*, 2012; Kim *et al.*, 2012). Thus, it is unlikely that Paneth cells are the sole source of Notch ligand.

Lineage marking cells with a Notch ligand *Dll1-CreERT* transgene showed expression in a subset of secretory progenitor cells, suggesting that committed TA cells may also be a source of Notch ligand for the GI stem cell (van Es *et al.*, 2012). Furthermore, the colon, which is devoid of Paneth cells, requires Notch signaling for stem/progenitor cell proliferation and differentiation, suggesting that Paneth cells are not the sole source of Notch ligand, although a Paneth-like goblet cell has been postulated to serve this function in the colon (Rothenberg *et al.*, 2012). Notch regulation of colonic stem cells is presumed to be

similar to small intestine. However, because most studies have not included colonic stem cells in their analysis this issue has not been investigated in depth.

Many studies have suggested that Notch signaling is crucial for CBC maintenance, with Notch pathway inhibition resulting in reduced stem cell proliferation. Global Notch inhibition via pharmacologic blockade of the gamma-secretase complex led to an overall reduction in intestinal proliferation, and more specifically, reduced proliferation and fewer LGR5-expressing CBCs (van Es *et al.*, 2005b; Pellegrinet *et al.*, 2011; VanDussen *et al.*, 2012). Consistent with the observed lineage tracing from CBCs expressing the NOTCH1 and NOTCH2 receptors (Fre *et al.*, 2011; Pellegrinet *et al.*, 2011), inhibition of both NOTCH1 and NOTCH2 receptor signaling via treatment with neutralizing antibodies or by genetic deletion resulted in reduced progenitor cell proliferation (Riccio *et al.*, 2008; Wu *et al.*, 2010; Tran *et al.*, 2013; Carulli *et al.*, 2015) and reduced LGR5⁺ CBC number (Carulli *et al.*, 2015). Furthermore these studies suggested that NOTCH1 is the dominant Notch receptor regulating CBCs (Carulli *et al.*, 2015). Moreover, genetic inhibition of other Notch pathway components, such as the DLL1 and DLL4 ligands (Pellegrinet *et al.*, 2011), the Notch receptor protease ADAM10 (Tsai *et al.*, 2014) or the NICD binding partner RBPJ (van Es *et al.*, 2005b) have been shown to reduce progenitor cell proliferation or loss of CBC stem cell function. Taken together, these data point to an essential role for Notch signaling to support intestinal CBCs.

Although much work has been devoted to understanding Notch regulation of CBC stem cells, much less is known about whether Notch plays a role in regulating QSCs. It was recently shown that global deletion of the QSC marker *Bmi1* results in modest reductions in intestinal length, progenitor cell proliferation and *Lgr5* expression, as well as a mild goblet cell hyperplasia, suggesting that BMI1 may interact with Notch signaling and that intestinal QSCs may contribute to intestinal epithelial cell homeostasis in the absence of injury (Lopez-

Arribillaga *et al.*, 2015). Moreover, this study showed that treatment of intestinal organoid cultures with the pan-Notch inhibitor DAPT resulted in reduced BMI1 expression, suggesting that Notch may regulate the BMI1⁺ QSC population. Furthermore, recent studies investigating the consequences of individual deletion of the *Notch1* and *Notch2* receptor genes in the intestinal epithelium showed impaired crypt regeneration post-irradiation injury, suggesting that Notch signaling may be required for recruitment of QSCs to active stem cells, or for functional restoration of the CBC stem cell compartment after QSC activation (Carulli *et al.*, 2015). These data highlight a potential role for Notch in regulating intestinal QSCs; however further studies are needed to define Notch pathway function for maintenance or activation of this stem cell population.

Notch regulation of intestinal cell differentiation

In the intestine, Notch signaling is thought to direct the differentiation of multipotential progenitors into absorptive enterocytes by inhibiting the program of secretory cell differentiation. Notch effects on cell fate have been primarily studied in mouse models where pathway disruption by pharmacologic inhibition or genetic deletion has been shown to induce a profound secretory cell hyperplasia (Fig. 3A) (Jensen *et al.*, 2000; van Es *et al.*, 2005b; Riccio *et al.*, 2008; Pellegrinet *et al.*, 2011). Conversely, studies of pathway activation in transgenic mice expressing a constitutively active form of NICD showed loss of secretory cell types (Fre *et al.*, 2005; Stanger *et al.*, 2005).

Regulation of secretory cell fate occurs via Notch pathway inhibition of the basic-helix-loop-helix (bHLH) transcription factor atonal homolog 1 (ATOH1, also called MATH1 in mouse) (reviewed in (Noah & Shroyer, 2013)). ATOH1 is expressed in secretory progenitors as well as differentiated secretory cell types and genetic deletion and activation studies in

mouse models have demonstrated that ATOH1 is the key transcriptional activator inducing the full program of secretory cell differentiation to generate goblet, Paneth and endocrine cells (Yang *et al.*, 2001; Shroyer *et al.*, 2007; VanDussen & Samuelson, 2010). The key Notch effector regulating *Atoh1* expression is thought to be the Notch target gene *Hes1*. Genetic deletion of *Hes1* in mouse results in excessive differentiation of secretory cell types, consistent with a Notch inhibition phenotype (Jensen *et al.*, 2000; Ueo *et al.*, 2012). Furthermore, Notch disruption results in decreased expression of *Hes1* and increased expression of *Atoh1*. The *Atoh1* promoter has been described to contain HES binding sites (Zheng *et al.*, 2011) and it is thought that Notch-induced HES1 acts as a transcriptional repressor to silence *Atoh1* transcription and suppress secretory cell fate.

Other than suppressing secretory cell fate through regulation of *Atoh1*, Notch signaling does not appear to play an active role in enterocyte differentiation. This was demonstrated by analysis of mice with both intestine-specific deletion of *Atoh1* and disruption of Notch signaling. The intestinal epithelium of these mice was filled with enterocytes, suggesting that Notch is not required to promote enterocyte differentiation (Kazanjian *et al.*, 2010; Kim & Shivdasani, 2011a).

Notch signaling can induce differential cell fate of adjoining cells. This process of Notch-induced lateral inhibition is thought to establish the homeostatic pattern of absorptive and secretory cell types in the intestine (reviewed in (Sancho *et al.*, 2015)). ATOH1-expressing progenitor cells express Notch ligand, which would activate Notch signaling in surrounding cells to turn off *Atoh1* and promote absorptive cell fate (Fre *et al.*, 2005; Kim & Shivdasani, 2011a). Thereby, secretory cell types in the small intestine are surrounded by absorptive enterocytes.

Several transcription factors have been identified to function downstream of ATOH1 to regulate specification of the mature secretory cell types (Fig. 4). Mouse loss-of-function

and overexpression studies have demonstrated that the bHLH transcription factor Neurogenin 3 (NEUROG3) directs enteroendocrine cell differentiation (Jenny *et al.*, 2002; Lee *et al.*, 2002; Lopez-Diaz *et al.*, 2007). GF11, a zinc-finger protein family member, has been shown to be a direct ATOH1 target gene that directs secretory progenitor cells to form goblet or Paneth cells (Shroyer *et al.*, 2005). Differentiation of goblet/Paneth progenitor cells into mature goblet cells also involves the transcription factor SPDEF (Noah *et al.*, 2010). It is currently unknown how progenitor cells are fated towards goblet or Paneth cells, but this process likely involves SPDEF, KLF4 (Zheng *et al.*, 2009), SOX9 (Bastide *et al.*, 2007; Mori-Akiyama *et al.*, 2007) FGF10 (Al Alam *et al.*, 2015) and Wnt (van Es *et al.*, 2005a).

A progenitor cell population expressing high levels of the Notch ligand DLL1 has been identified (van Es *et al.*, 2012; Buczacki *et al.*, 2013). These cells express low levels of stem cell markers and high *Atoh1*, characteristics of committed secretory progenitor cells. Lineage tracing studies showed that these DLL1⁺ cells labeled short-lived clones containing all 4 secretory cell types suggesting that they are multipotential secretory cell precursors (van Es *et al.*, 2012). Additionally, during radiation-induced loss of CBCs these committed progenitor cells exhibited plasticity by re-gaining stemness to regenerate the epithelium, thereby also functioning as QSCs (van Es *et al.*, 2012; Buczacki *et al.*, 2013). This finding supports the notion that TA progenitors can fill open niche spots left vacant when CBC stem cells are lost.

Notch regulation of gastric epithelial cell homeostasis

Gastric stem cells

While a large volume of literature exists regarding Notch pathway regulation of intestinal stem cells, much less is known about Notch regulation of gastric stem cells, largely

due to the paucity of molecular markers for stem cells in this tissue. The glandular stomach is divided into 2 regions: the corpus, which secretes gastric acid and digestive enzymes into the gastric lumen, and the more distal antrum, which secretes the hormone gastrin into the blood. Gastric stem cells exist in both regions to generate the various differentiated gastric lineages throughout adult life (Fig. 2B). However, there are distinct differences in stem/progenitor cell location as well as marker expression in these regions. In the corpus, proliferating cells (and presumably stem cells) are located in the middle of the corpus glands, while in the antrum, stem and TA progenitor cells are located at the gland base, in a pattern similar to that observed in the intestinal crypt (Fig. 2). In line with the similar patterning, the CBC marker LGR5 has been identified as a marker of antral stem cells, based on lineage tracing analysis and initiation of organoid cultures from FACS-sorted LGR5-GFP stem cells (Barker *et al.*, 2010). Approximately 8 LGR5⁺ stem cells are present in each antral gland (Leushacke *et al.*, 2013).

In contrast to the antrum, corpus stem cell markers are less well established. Although LGR5 marks a precursor to the corpus stem cell, adult corpus stem cells do not express this marker (Barker *et al.*, 2010). SOX2 has been shown to be expressed in both corpus and antral stem cells (Fig. 2B), with long-lived lineage stripes observed in both compartments after activation of a reporter gene by the Sox2-CreER^{T2} driver (Arnold *et al.*, 2011). However, this gene is also expressed in differentiated gastric cell types in addition to stem cells. Importantly, in contrast to LGR5, SOX2 does not label ISCs, thus allowing genetic manipulation of gastric epithelial cells without affecting the intestine. Markers described for facultative gastric stem cells include *Villin*, which marks a rare quiescent antral stem cell (Qiao *et al.*, 2007), and *Troy*, which is expressed in a small population of differentiated chief cells (Stange *et al.*, 2013). Both of these markers are expressed in non-proliferating gastric cells that are capable of expanding and replenishing entire gastric glands

after injury, similar in function to QSCs in the intestine (Fig. 2). Additionally, *Lrig1*, a reported intestinal QSC marker, also marks a population of gastric stem cells in both corpus and antrum, although this marker also appears to label differentiated cells in both regions (Powell *et al.*, 2012). Clearly more work is needed to identify specific markers of gastric stem cells that are not expressed in differentiated gastric cell types or in intestinal stem cells.

Notch regulation of gastric stem cell proliferation

Notch regulation of adult gastric stem cells has only recently been established. Lineage tracing from cells with active NOTCH1 signaling has been demonstrated in antral stem cells (Demitrack *et al.*, 2015; Gifford *et al.*, 2016). Functional studies have shown that similar to its effect in the intestine, Notch signaling promotes gastric epithelial cell proliferation. This was demonstrated by the reduction in proliferation observed after pharmacologic blockade of the gamma-secretase complex in both corpus (Kim & Shivdasani, 2011b) and antrum (Kim & Shivdasani, 2011b; Demitrack *et al.*, 2015; Gifford *et al.*, 2016). Importantly, examination of the LGR5-expressing antral stem cells after Notch manipulation showed alterations in stem cell function. Notch inhibition was found to reduce stem cell proliferation, while Notch activation by genetic expression of NICD caused increases in proliferation and stem cell function (Demitrack *et al.*, 2015). This study also showed that gastric organoids were similarly affected by Notch, with decreased efficiency of establishment and organoid growth with Notch pathway inhibition, and increased efficiency and growth with Notch activation. Over time, chronic NICD expression in parietal progenitor cells of the corpus (Kim & Shivdasani, 2011b) or LGR5⁺ antral stem cells (Demitrack *et al.*, 2015) resulted in the formation of hyperplastic polyps, further establishing Notch as a crucial pathway regulating proliferation.

Similar to intestine, NOTCH1 and NOTCH2 appear to be the key receptors mediating Notch effects in the antrum. Specific blockage of these two receptors by treatment with inhibitory antibodies led to reduced proliferation and alteration of differentiation similar to global Notch inhibition with a gamma-secretase inhibitor (Gifford *et al.*, 2016). Furthermore, NOTCH1 appears to be the dominant receptor regulating gastric stem cells, similar to what has been shown in intestine (Carulli *et al.*, 2015; Gifford *et al.*, 2016). Analysis of gastric organoids established from human antral tissue confirmed that Notch signaling is essential to maintain human gastric stem cells through NOTCH1 and NOTCH2 signaling (Gifford *et al.*, 2016). Thus human gastric stem cells appear to be similarly regulated as the mouse, validating the mouse as an effective model to study Notch regulation.

Notch regulation of gastric stem cell differentiation

The Notch pathway has also been shown to regulate gastric epithelial cell differentiation. However, in contrast to intestine, where Notch is key to cell fate determination (Fig. 3A), in the stomach Notch appears to similarly affect all gastric lineages. These studies largely focused on the antrum, where cellular turnover is relatively rapid, occurring within a week, rather than the corpus where cell turnover ranges from a few days (surface mucous cells) to 6 months (chief cells). Notch inhibition increased antral cellular differentiation, including surface mucous, deep mucous and endocrine cells while Notch activation resulted in decreased numbers of all lineages (Fig. 3B) (Demitrack *et al.*, 2015; Gifford *et al.*, 2016). These findings were demonstrated in both *in vivo* mouse models and *ex vivo* gastric organoid cultures.

Although a master transcriptional regulator of cellular differentiation in the stomach has not been identified, transcription factors that regulate differentiation of mature antral

lineages are known. As such, Notch signaling affects the expression of these factors in line with the cell lineage changes, including *FoxQ1* (surface mucous cells), *Spdef* (deep mucous cells) and *Neurog3* (endocrine cells), although whether this is direct or indirect is not known (Fig. 4) (Demitrack *et al.*, 2015). In sum, these data suggest that active Notch signaling promotes proliferation and represses cellular differentiation in the antrum.

Comparison to Notch effects in the intestine

Notch regulation of gastric and intestinal epithelial cell homeostasis has many parallels, yet also distinct differences. Notch signaling promotes stem cell proliferation in both stomach and intestine, serving as an essential niche factor for the active LGR5⁺ stem cell. In contrast, regulation of cell fate is fundamentally different in these two tissues. In the gastric antrum, Notch appears to affect choice between stem cell proliferation or differentiation to maintain homeostasis, and does not promote the differentiation of one lineage at the expense of another as has been established in intestine via regulation of ATOH1. More work is needed to determine whether a Notch-dependent master transcriptional regulator, such as another bHLH family member, exists for differentiation of gastric epithelial lineages. However, it is important to note that in contrast to the intestine the adult stomach only contains secretory cell types.

Notch and antral tissue expansion

In the gastric antrum, constitutive Notch activation has been shown to induce gland fission, a tissue growth phenomenon thought to be associated with increased stem cell number (Demitrack *et al.*, 2015). Gland fission is a process by which a single gland

bifurcates, or splits, into 2 separate glands (Lee, 1985). It is an essential part of postnatal stomach growth during the time that gastric glands are undergoing cellular maturation (Nomura *et al.*, 1998; Keeley & Samuelson, 2010). Fission is rarely observed in the adult stomach. Recent studies have shown that NICD activation in LGR5⁺ antral stem cells induces antral gland fission and eventual formation of hyperproliferative undifferentiated polyps (Demitrack *et al.*, 2015). In the human stomach, cancer-initiating mutations in gastric stem cells have been shown to spread via gland fission (McDonald *et al.*, 2008); however whether Notch plays a role in this process in human is not known.

Notch and gastrointestinal disease

Dysregulation of Notch signaling has been implicated in tumorigenesis of a variety of tissues, particularly in the hematopoietic system where activating mutations in the NOTCH1 receptor lead to T cell acute lymphoblastic leukemia (Demarest *et al.*, 2008). Recent studies also point to a link between overexpression of Notch pathway components and colorectal cancer (Reedijk *et al.*, 2008; Fre *et al.*, 2009; Chu *et al.*, 2010; Kazanjian & Shroyer, 2011; Guilmeau, 2012); these studies have been nicely summarized in a recent review (Noah & Shroyer, 2013).

Much less is known about the role of Notch in gastric cancer, although correlative studies between high NOTCH1 and NOTCH2 expression and gastric cancer morbidity have been published (Yeh *et al.*, 2009; Hsu *et al.*, 2012; Bauer *et al.*, 2015). The observation of NICD-induced polyps in mouse genetic models suggests that Notch pathway activation induces aberrant stem and progenitor cell proliferation, which could then initiate and/or sustain gastric tumors (Kim & Shivdasani, 2011b; Demitrack *et al.*, 2015). It has also been postulated that Notch may be involved in a pre-cancerous, metaplastic condition in the

stomach known as spasmodic polypeptide-expressing metaplasia (SPEM) (Mutoh *et al.*, 2006), which typically results from loss of parietal cells and chronic inflammation. ATOH1, which is normally absent from the normal mouse and human stomach, has been shown to be upregulated in a mouse model of intestinal metaplasia as well as in the metaplastic human stomach (Mutoh *et al.* 2006). Whether ectopic activation of ATOH1 is due to the intestinalization of the stomach in these situations or includes a role for Notch in promoting these metaplastic conditions remains to be elucidated.

Conclusions and future directions

The Notch pathway is crucial for gastrointestinal epithelial cell homeostasis, regulating stem cells in both the stomach and intestine. Notch is required for stem cell maintenance and manipulation of Notch signaling affects stem cell function and number. Notch inhibition is associated with reduced stem cell proliferation and activity while Notch activation is associated with increased stem cell proliferation and activity. Together the findings suggest that Notch is a key niche factor regulating stem cell self-renewal in gastrointestinal tissues.

Our understanding of Notch regulation of gastrointestinal stem cells has primarily been developed through extensive studies of mouse pharmacologic and genetic models. With the recent development of methods to grow epithelial organoids from primary human tissue there will be future opportunities to directly study human gastrointestinal stem cells. Notch pathway regulation of intestinal cell homeostasis is highly conserved, with parallel functions reported in other organisms, such as *Drosophila* (Micchelli & Perrimon, 2006) and zebrafish (Yang *et al.*, 2009), suggesting that this pathway will also be a crucial regulator in human. Indeed, recent analysis of human gastric organoids demonstrated that Notch

regulates human antral stem cells similar to what has been shown in the mouse (Gifford *et al.*, 2016). Describing the key Notch pathway components and molecular mechanisms driving human gastrointestinal stem cell function will be important to further our understanding of human gastrointestinal epithelial cell maintenance and repair.

Some timely unanswered questions that need to be addressed to further our understanding of Notch regulation of mouse and/or human stem cells include:

- How does Notch regulate gastrointestinal stem cell number?
- Does Notch regulate the “quiescent” stem cell population?
- Which cells present Notch ligand to regulate stem cell function? Are there niche cells in the stomach equivalent to Paneth cells in the intestine?
- Which Notch ligands function in the stomach?
- Does Notch regulate differentiation of cells in the gastric corpus?
- What are the mechanisms of Notch regulation of human gastrointestinal stem cells?

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Figure Legends

Abstract Figure: Notch regulation of intestinal epithelial cell homeostasis. (A) During homeostatic conditions, Notch maintains the intestinal stem cell (SC) pool and promotes proliferation of a multi-potential (MP) progenitor cell that will further differentiate into an absorptive progenitor (AP) or secretory progenitor (SP) cell in the intestine. Therefore, Notch is required to maintain the balance of stem cell proliferation and differentiation. (B) During Notch inhibition, stem cell proliferation is reduced and progenitor cells are fated towards the secretory lineage. This results in a net reduction in proliferation and increased secretory cell differentiation. (C) In contrast, Notch activation leads to expansion of the stem cell compartment, resulting in increased stem cell proliferation and an overall reduction in epithelial cell differentiation.

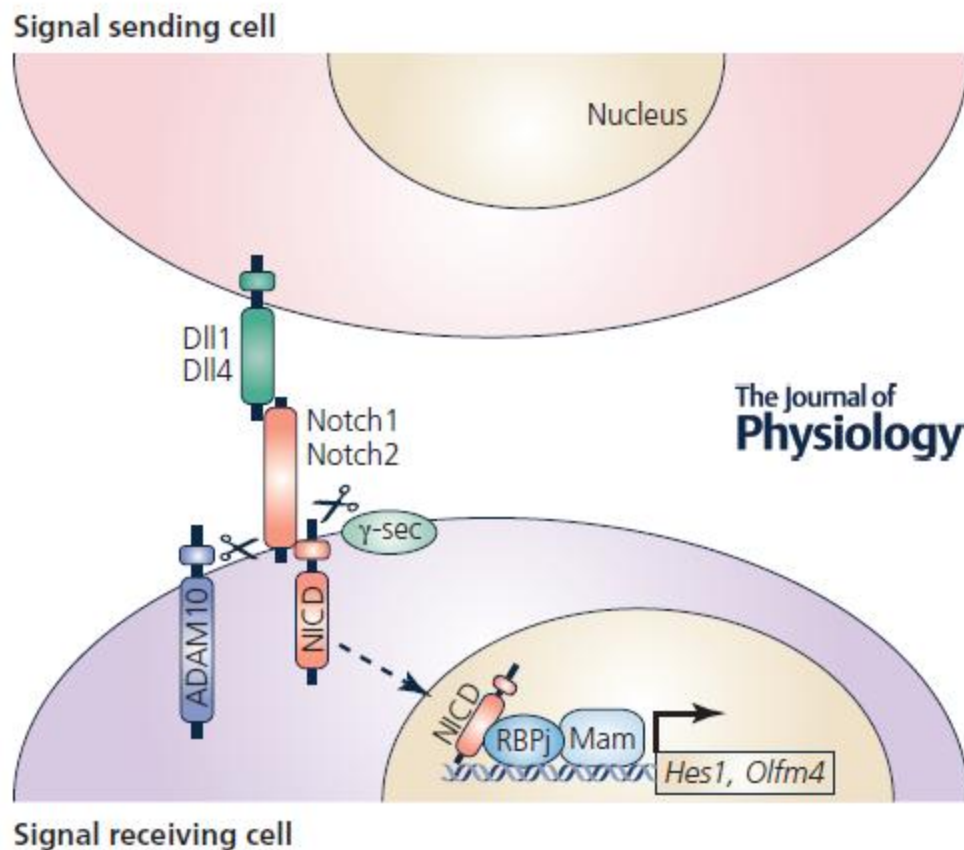


Figure 1: Notch signaling in the intestinal epithelium. Transmission of a Notch signal involves engagement of two cells: a signal-sending cell (*top*) that expresses Notch ligand (DLL1 and DLL4 in intestine), and a signal-receiving cell (*bottom*) that expresses Notch receptor (NOTCH1 and NOTCH2 in intestine and stomach). After ligand/receptor engagement, the Notch receptor is proteolytically cleaved: first by ADAM10, which releases the extracellular domain, and then by the gamma-secretase complex, which cleaves the receptor at the cell membrane to release the Notch Intracellular Domain (NICD). NICD translocates to the nucleus where it recruits a transcriptional co-activator complex that activates downstream transcription of Notch target genes, such as *Hes1* and *Olfm4*.

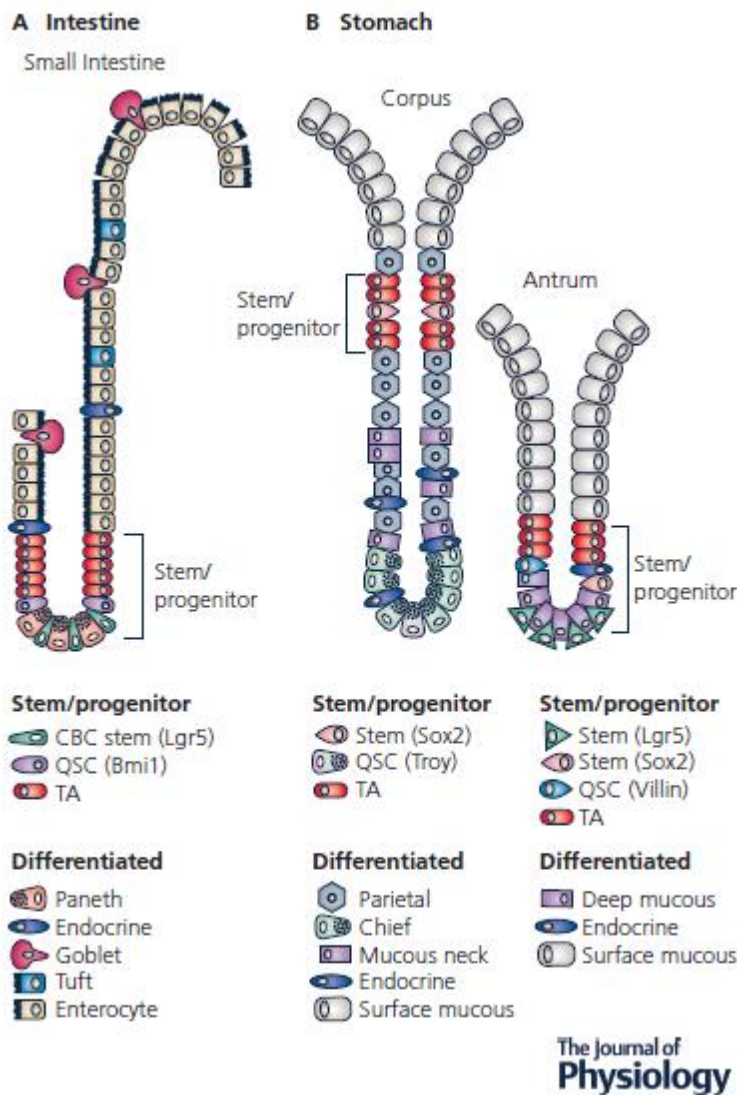


Figure 2: Stem and differentiated cells of the small intestine and stomach. Schematic of the cellular composition of small intestinal crypt/villus units and gastric corpus and antral gland units, highlighting selective markers that are proposed to define distinct stem cell populations. In both stomach and intestine, stem cells proliferate to form transit-amplifying (TA) progenitors which differentiate into the mature cell types of each tissue, as indicated. The mature cell types are replaced every several days (intestine and antrum) to several months (corpus). (A) In the intestine, active LGR5-expressing CBC stem cells are located at the crypt base, intercalated between Paneth cells. A quiescent BMI1-marked stem cell (QSC) population is located approximately 4 cell positions from the crypt base. (B) In the stomach, stem and progenitor cells are located in the upper mid-region in corpus and at the gland base in antrum. LGR5 marks active stem cells in the antrum, but not corpus. Other potential gastric stem cell markers are discussed in the text.

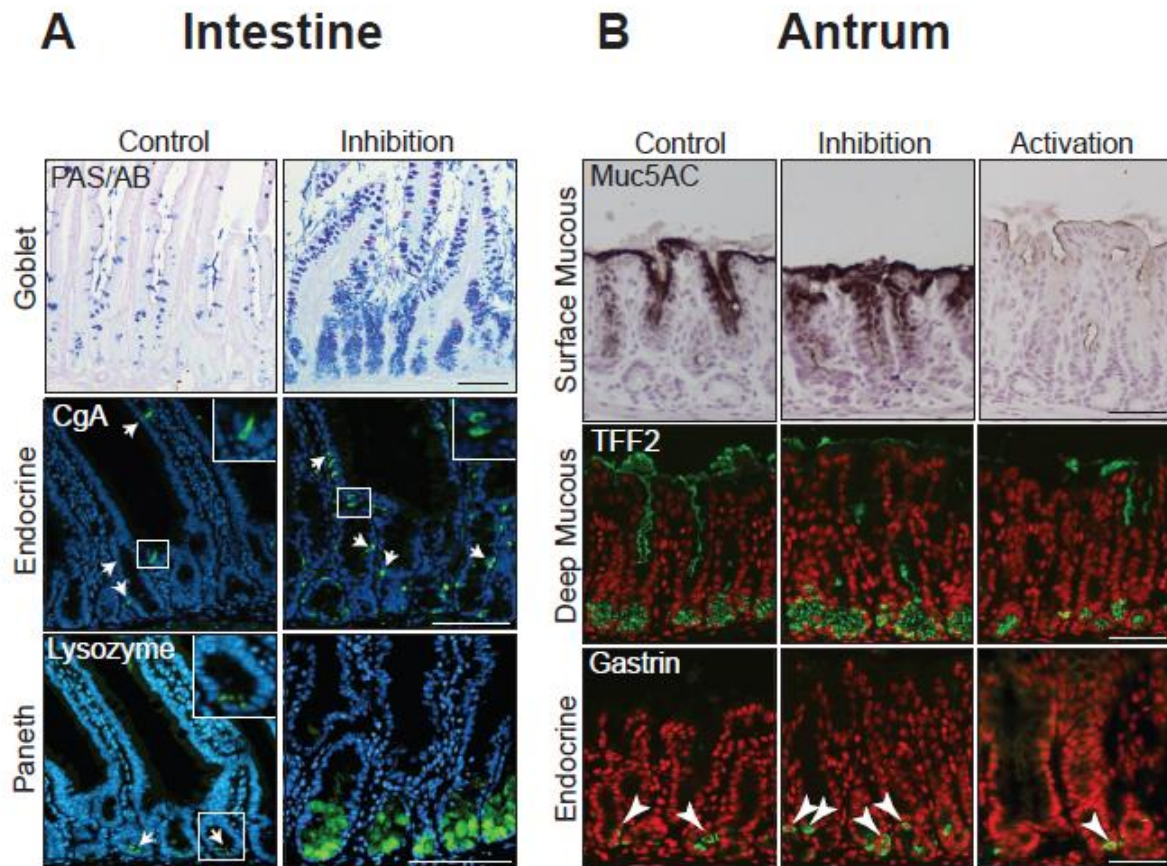


Figure 3: Notch regulates gastrointestinal epithelial cell differentiation. (A) Inhibition of Notch with the gamma-secretase inhibitor DBZ induced expansion of all intestinal secretory cell lineages. Analysis of intestinal differentiated lineages by histological analysis, including goblet cells (PAS/AB), endocrine cells (chromogranin A; CgA) and Paneth cells (lysozyme) in control or Notch-inhibited mouse duodenum. Arrows indicate CgA⁺ endocrine and Lysozyme⁺ Paneth cells. Insets are magnified from lower-powered boxed regions. (B) Analysis of gastric antral epithelial cells, including surface mucous cells (Muc5AC), deep mucous cells (TFF2) and endocrine cells (gastrin) in control, Notch-inhibited and Notch-activated (*Lgr5-CreER^{T2}; ROSA^{NICD}*) mice. Notch inhibition induced differentiation of all three epithelial cell types, while Notch activation induced a generalized reduction in differentiation. Arrowheads indicate gastrin⁺ endocrine cells. Scale bars: 50 (B) or 100 (A) μ m. Figure modified from (VanDussen *et al.*, 2012) and (Demitrack *et al.*, 2015).

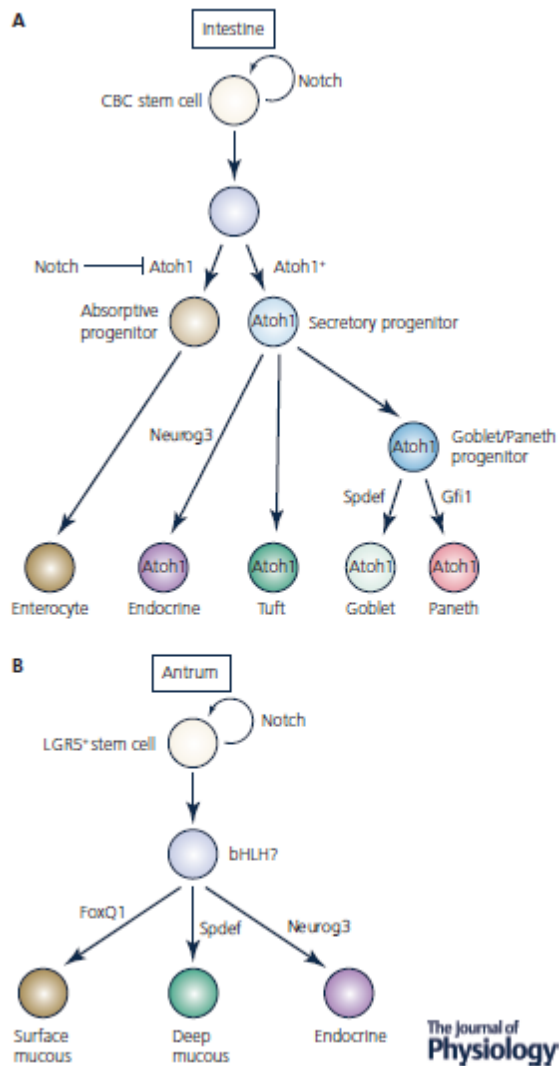


Figure 4: Model of Notch regulation of intestinal and gastric epithelial cell differentiation. Notch is required for renewal of both (A) intestinal and (B) gastric antral stem cells. In the intestine, Notch signaling represses the bHLH transcriptional regulator *Atoh1* to form absorptive enterocytes. *ATOH1*⁺ secretory progenitor cells are fated towards 1 of 4 mature secretory cell types: endocrine, tuft, goblet or Paneth. Note that SPDEF and/or GFI1 regulate goblet and Paneth cell differentiation while *Neurog3* induces endocrine cell differentiation. A master transcriptional regulator, possibly a member of the bHLH family, has not been identified for the gastric antrum, although transcription factors that promote differentiation of each mature lineage are known. In the stomach, recent studies suggest that Notch functions to regulate gastric stem cell proliferation versus differentiation, and not choice between different differentiated cell types.