

**NOTCH1 AND NOTCH2 RECEPTORS REGULATE HUMAN AND MOUSE GASTRIC
EPITHELIAL CELL HOMEOSTASIS**

by

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This thesis is dedicated to my family: Mom, Dad, Katie, and Dan. For your continuous love, support, and encouragement.

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ABSTRACT

NOTCH1 AND NOTCH2 RECEPTORS REGULATE HUMAN AND MOUSE GASTRIC EPITHELIAL CELL HOMEOSTASIS

by

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Chair: Linda C. Samuelson

The gastric epithelium undergoes constant turnover that is maintained by a population of gastric stem cells. Gastric stem cells are under the regulation of multiple signaling pathways to promote proper epithelial homeostasis. Previous studies have shown that the Notch signaling pathway plays a crucial role in regulating epithelial differentiated cell fate, stem cell function, and epithelial cell proliferation in the stomach. My thesis work has focused on identifying the mechanisms by which Notch signaling regulates gastric epithelial cell homeostasis.

I identified Notch1 and Notch2 as the key receptors contributing to the regulation of gastric epithelial cells by Notch. Using inhibitory antibodies targeting Notch1 and Notch2, I observed a marked reduction in proliferation of both corpus and antral epithelial cells that mimicked the reduced proliferation observed with global Notch inhibition. Inhibition of Notch1 or Notch2 signaling led to an intermediate reduction in

proliferation in both regions of the glandular stomach. In the antrum, inhibition of both receptors resulted in a general increase in expression of markers of differentiated cells, including enteroendocrine, surface mucous, and deep mucous cells. Inhibition of both receptors also led to increased secretory granules in antral cells and expression of secretory products from other regions of the gastrointestinal tract, including the corpus and intestine.

To investigate if Notch signaling is intrinsic to the epithelium, I refined the conditions for gastric organoid establishment from mouse and human antrum and corpus tissue. In mouse and human antral and corpus organoids, inhibition of Notch1 and Notch2 resulted in a reduction of organoid growth similar to that seen with global Notch inhibition. In corpus organoids, inhibition of either Notch1 or Notch2 resulted in an intermediate disruption of organoid growth. However, in antral organoids, inhibition of Notch1 mimicked growth similar to that seen with global inhibition, suggesting that Notch1 may play a more significant role in antral organoid growth than Notch2.

In summary, my thesis work has expanded the understanding of the role of Notch in gastric epithelial homeostasis. I have illustrated an important role for the Notch1 and Notch2 receptors in regulating gastric epithelial proliferation and differentiation *in vivo* and *in vitro*.

CHAPTER 1

INTRODUCTION

Stem cells are undifferentiated cells capable of self-renewal and division into multiple differentiated cell types. Stem cells are key cells to maintain tissue homeostasis in all multicellular organisms. From initial development from a single pluripotent embryonic stem cell through adult organ-specific maintenance by tissue-specific stem cells, an organism's viability depends on the proper functioning of these stem cells. The balance of proliferation and differentiation is key to not only maintaining proper function, but also to prevent unchecked stem cell proliferation that may contribute to disease.

The epithelium of the adult stomach is maintained through the proper functioning of stem cells, which proliferate and differentiate into all glandular cell types. The homeostasis of these stem cells is crucial to produce adequate numbers of cells to maintain epithelial integrity. Unchecked proliferation of gastric stem cells can contribute to hyper-proliferative states, such as gastric cancer. Understanding how these gastric stem cells function not only contributes to the advancement of stem cell physiology, but also provides a mechanism of how aberrant stem cell activity can contribute to disease development.

A key question in gastric stem cell biology is how signaling pathways play a role in maintaining stem cell homeostasis. The Notch signaling pathway is a conserved cell-cell signaling pathway that is crucial for maintenance of skin, hematopoietic, intestinal, and other tissue-specific stem cells¹. In the intestine, Notch is a regulator of stem and proliferative cell maintenance and directs cell fate decisions between absorptive versus secretory cell types²⁻⁷. The components of the Notch pathway involved in intestinal regulation have been uncovered, with the Notch receptors Notch1 and Notch2 and Notch ligands Delta-like1 (Dll1) and Delta-like 4 (Dll4) maintaining intestinal epithelial homeostasis^{5,6,8,9}. Notch is also a key regulator of gastric stem and epithelial cell homeostasis^{10,11}. However, the Notch components regulating gastric stem and epithelial cells have not been uncovered. The goal of this thesis is to investigate the mechanism of Notch regulation of gastric epithelial cell homeostasis. Using transgenic mouse models, pharmacologic inhibition, and *in vitro* organoid culture, my studies addressed the role of the Notch receptors in regulating gastric corpus and antral epithelial cell homeostasis in mouse and human.

This chapter covers three broad topics: (1.1) an overview of gastric stem and epithelial cells and signaling pathways involved in maintaining homeostasis, (1.2) the Notch signaling pathway, and (1.3) the current understanding of the role of Notch signaling on gastrointestinal stem and epithelial cell maintenance. Finally, this chapter concludes with an overview of the experimental approaches and main findings of the thesis (1.4).

1.1: GASTRIC STEM AND EPITHELIAL CELLS

Anatomy and compartments

The stomach is a muscular organ located in the gastrointestinal tract that is important for proper digestion. The mouse stomach can be divided into three parts: the forestomach and the glandular corpus and antrum. The glandular stomach regions have distinct functions. The corpus is responsible for acid secretion while the antrum is responsible for mucous and hormone secretion. A transition zone exists between the corpus and antrum and contains features of both regions. The simple columnar glandular epithelium is organized into multiple gastric units, or glands, which are invaginations of epithelial glands into the underlying mesenchymal tissue. The epithelium of the stomach is highly dynamic with continuous proliferation, migration, differentiation, and apoptosis. Along with different functions, the architecture and epithelial cell types differ in the corpus and antrum.

Corpus gland structure and cell types of the mouse stomach

The corpus is composed of long tubular glands that open into the stomach lumen (Fig 1-1). Corpus glands can be divided into four regions: the pit, isthmus, neck, and base. Stem cells located in the isthmus divide to produce new stem cells or transit amplifying (TA) cells. TA cells are undifferentiated cells that divide more frequently than stem cells to increase the overall cell population in a gastric gland. TA cells include preparietal, prepit, or preneck cells¹²⁻¹⁴. These TA cells are able to migrate

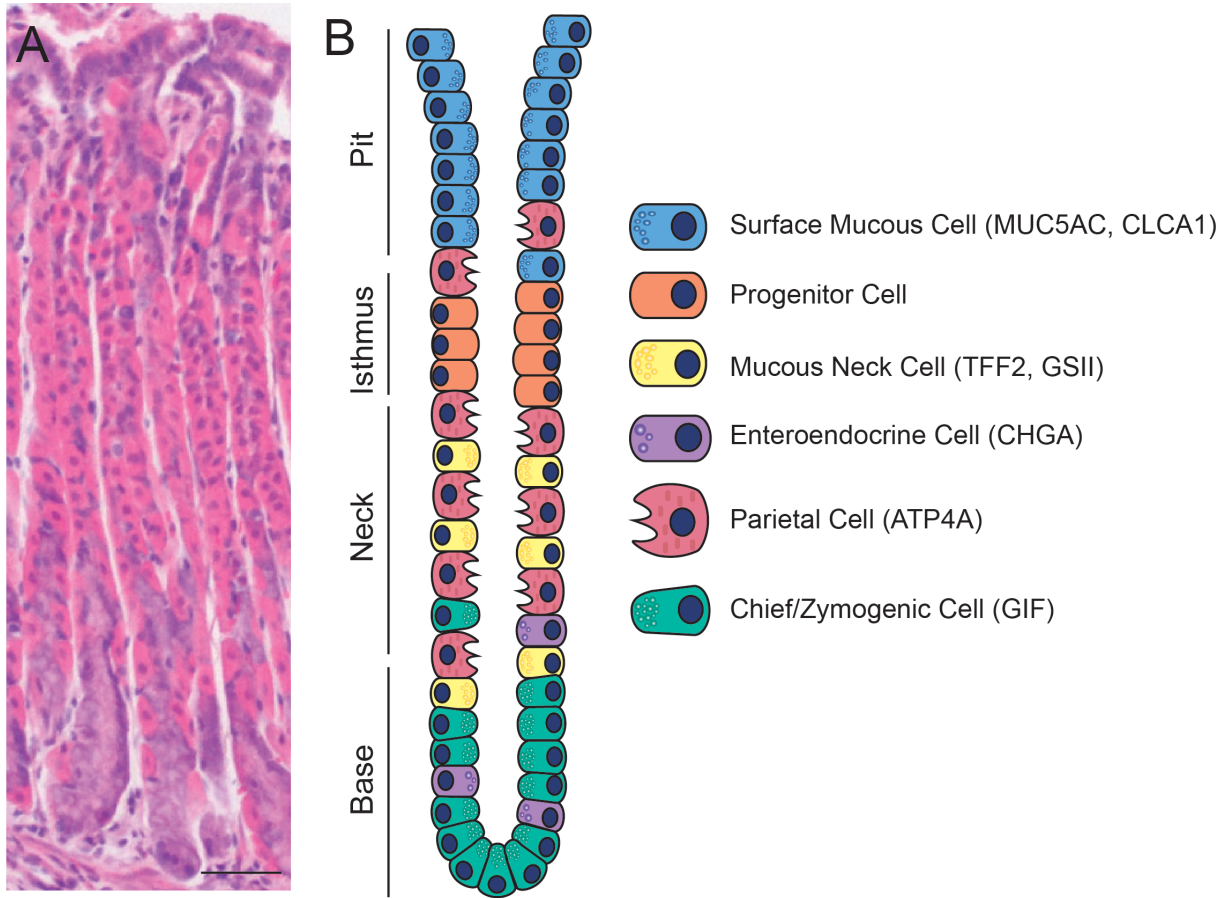


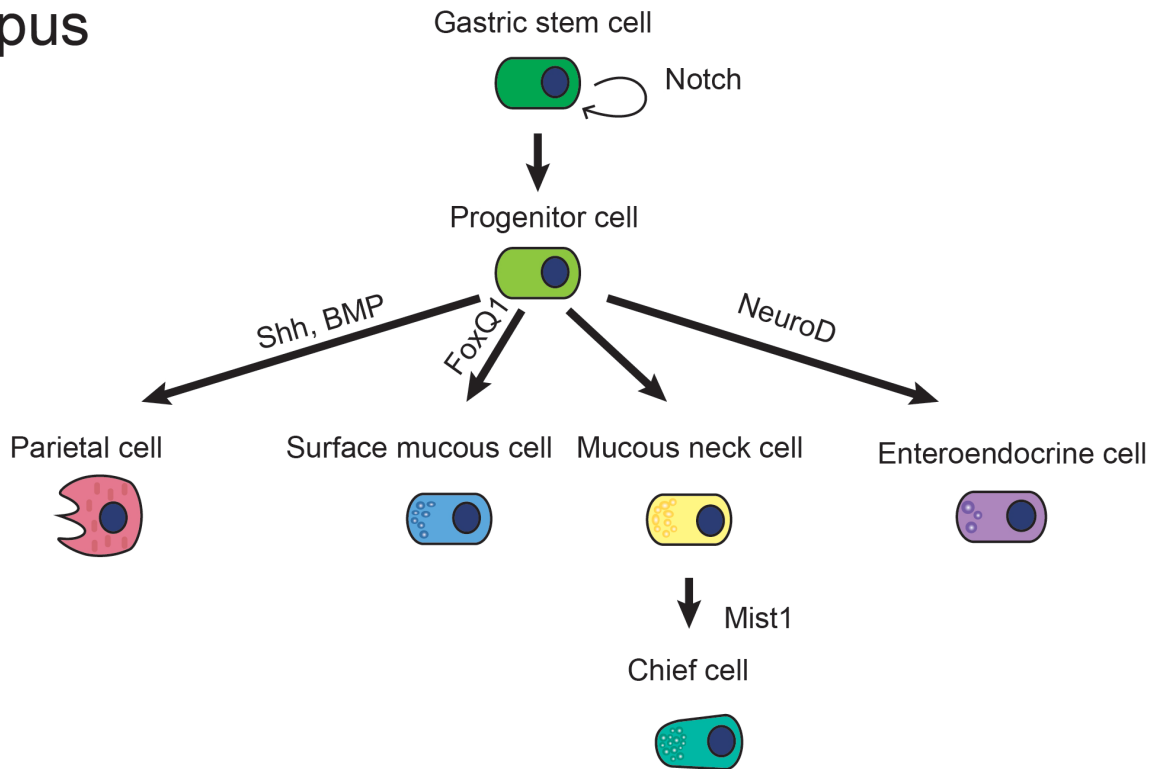
Figure 1-1. Mouse corpus gland. (A) Histological section of mouse corpus gland stained with haematoxylin and eosin. (B) Cartoon depiction of mouse corpus gland with various cell types identified with specific markers used in this study to identify the differentiated cell types. Mucin 5AC (MUC5AC), chloride channel accessory 1 (CLCA1), trefoil factor 2 (TFF2), griffonia simplicifolia II (GSII), chromogranin A (CHGA), H/K ATPase α subunit (ATP4A), gastrin intrinsic factor (GIF). Scale = 25 μ m.

bidirectionally to differentiate and populate all four regions of the corpus gland (Fig 1-1, 1-2).

Surface mucous cells secrete mucous and are primarily located in the pit segment of the corpus gland. Surface mucous cells contain densely packed mucous granules and express mucin 5AC (MUC5AC), gastrophilin-1, and trefoil factor family 1 (TFF1); they are generated from prepit progenitor cells^{12,15}. The Hedgehog pathway may play a role in surface mucous cell generation, with a loss of surface mucous cells in primary mouse gastric epithelial cultures treated with cyclopamine, a Hedgehog pathway inhibitor¹⁶. The transcription factor *FoxQ1* has been shown to be required for surface mucous cell formation, with a loss of *FoxQ1* leading to reduced surface mucous cell numbers¹⁷. Surface mucous cells turn over every 2-3 days¹⁴.

Parietal cells are the acid-secreting cell type of the stomach and can be located in any region of the corpus gland. Parietal cells contain a highly dense canalicular network from which acid is secreted into the lumen of the stomach¹⁸. Parietal cells express H/K ATPase, parathyroid hormone-like hormone (PTHLH), and ADP-ribosylation factor 1 (ARF1)¹⁵. The Hedgehog pathway and epidermal growth factor (EGF) are thought to play a key role in parietal cell differentiation, as cultures of canine parietal cells stimulated with either EGF or sonic hedgehog increased expression of H/K ATPase¹⁹. The bone morphogenic pathway (BMP) also plays a role in parietal cell differentiation; a mouse model expressing the BMP antagonist noggin in parietal cells exhibited loss of parietal cells and increased proliferation²⁰. Parietal cells are generated from preparietal cells and turnover every 54 days¹⁴.

Corpus



Antrum

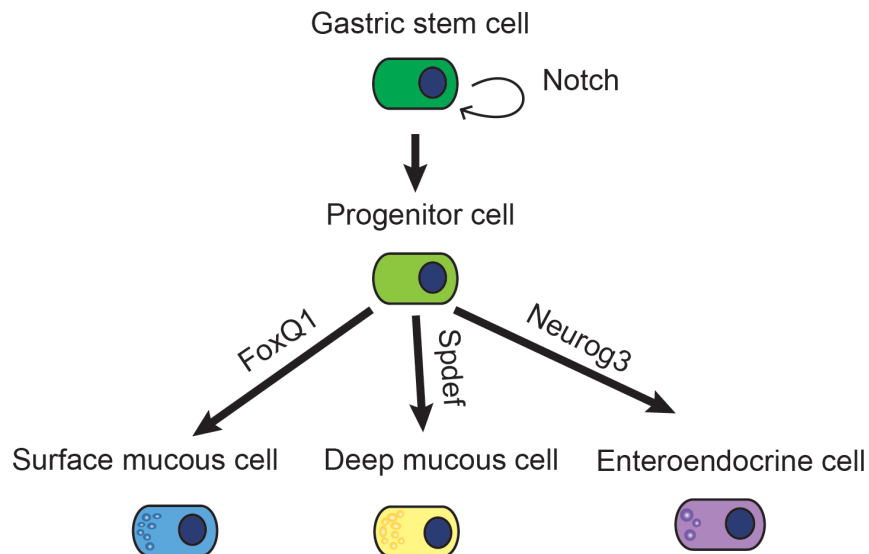


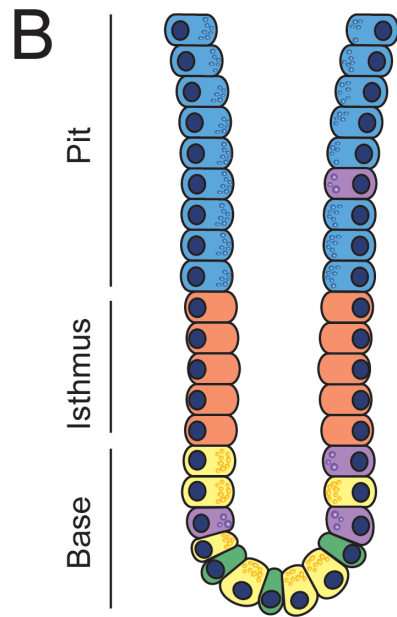
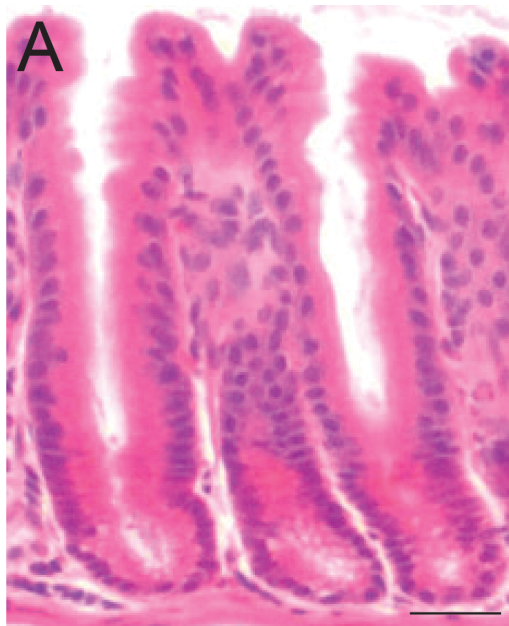
Figure 1-2. Schematic of gastric epithelial differentiation. Schematic depicting the key pathways or transcription factors for development of differentiated cell types of the corpus and antral epithelium. Key transcription factors described in text.

The pre-neck transition cells give rise to mucus-secreting mucous neck cells in the neck region of the corpus gland, and eventually mature into digestive enzyme secreting chief cells²¹. *Mist1* is a key transcription factor regulating this maturation. A genetic loss of *Mist1* leads to structural abnormalities in chief cells and an increase in pre-neck transition cells²¹. Chief cells are the longest-lived differentiated cell type of the stomach, with a half life of 194 days²². Mucous neck cells express trefoil factor family 2 (TFF2) while chief cells express pepsinogen C (PGC) and gastric intrinsic factor (GIF)²³⁻²⁵.

Scattered throughout the corpus gland are enteroendocrine cells, which are broadly marked by chromogranin A (CHGA)²⁶. The histamine-secreting enterochromaffin-like (ECL) cells are the primary enteroendocrine cell type in the corpus, but there are also ghrelin-, somatostatin-, and serotonin-secreting cells²⁷. There has not been a significant amount of work characterizing these cell types, but approximately 50% of CHGA-expressing enteroendocrine cells in the corpus, including all ECL and serotonin-expressing cells, originate from *NeuroD*-expressing cells²⁸.

Antral gland structure and cell types of the mouse stomach

In the antral region, key features of gland architecture and cellular make-up differentiate this region from the corpus. Shorter gastric units resemble intestinal crypts, with stem cells housed in the base of the gland (Fig 1-3)²⁹. These antral stem cells (marked by leucine-rich repeat-containing G-protein coupled receptor 5 [LGR5]) differentiate into surface mucous, deep mucous and enteroendocrine cells (Fig 1-2). The antral epithelium undergoes turnover much faster than the corpus, with complete








-  Surface Mucous Cell (MUC5AC, CLCA1)
-  Progenitor Cell
-  Deep Mucous Cell (TFF2, GSII)
-  Enteroendocrine Cell (CHGA, GAST)
-  Stem Cell (LGR5)

Figure 1-3. Mouse antral gland. (A) Histological section of mouse antral gland stained with haematoxylin and eosin. (B) Cartoon depiction of mouse antral gland with various cell types identified with specific markers used in this study to identify the differentiated cell types. Mucin 5AC (MUC5AC), chloride channel accessory 1 (CLCA1), trefoil factor 2 (TFF2), griffonia simplicifolia II (GSII), chromogranin A (CHGA), gastrin (GAST), leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5). Scale = 25 μ m.

turnover in 7-10 days³⁰. In the antrum the majority of enteroendocrine cells are descendants of *Neurog3* expressing progenitors³¹. The transcription factor SPDEF is required for mucous neck cell differentiation in the antrum, suggested from studies showing genetic mouse models lacking *Spdef* had impaired terminal maturation of deep mucous cells and reduced numbers of secretory granules³². Similar to the corpus, FOXQ1 is a necessary transcription factor for proper differentiation of deep mucous cells¹⁷.

Features of the human stomach

The human stomach exhibits some differences compared to the mouse stomach³³. The human stomach lacks a forestomach, but does include a glandular cardia region around the gastroesophageal junction. Human gastric glands are typically branched with multiple glands leading to a single luminal pit. While the mouse stomach is characterized by the restriction of parietal cells to the corpus, parietal cells in the human stomach are seen throughout the corpus and in over 50% of antral glands³³. Human antral glands are subgrouped into three types: oxyntic-type (containing parietal and chief cells), antral type (containing G-cells and TFF2-positive mucous cells) and mixed-type (containing both parietal and G-cells). The human corpus is distinguished by the expression of ghrelin, and the antrum by gastrin³³.

Gastric stem cells

The homeostasis of adult tissues relies on small populations of resident stem cells. These specialized cells are able to maintain themselves over long periods of time

(self-renewal) and generate all differentiated cell types of the resident tissue (multipotency). The balance between stem cell self-renewal and differentiation must remain under tight control to maintain proper development and avoid uncontrolled proliferation that may lead to cancer. Stem cells exist in two states: quiescent and active³⁴. Active stem cells undergo frequent division and contribute to normal epithelial homeostasis. Quiescent stem cells divide infrequently and typically contribute to epithelial cell renewal under injury conditions where active stem cells may be damaged. Both active and quiescent stem cells have been defined for the gastric epithelium^{29,35-37}.

Stem cells reside in specific microenvironments (niches) that support crucial stem cell maintenance. The localization of gastric stem cells was first identified in the 1940s, when ³²P nucleotides were incorporated into nuclei of dividing cells, which appeared in the isthmus region of corpus glands³⁸. The investigators concluded that this region was the site of cellular renewal in undamaged tissue. These cells were characterized as “nondifferentiated cells”, with a high nucleus-to-cytoplasm ratio, open chromatin, lack of granules, and many free ribosomes with few mitochondria³⁹. Similar cells have been localized in the isthmus region of the antrum²⁹.

The concept of gland monoclonality, or the derivation of all mature lineages in a gland from a single stem cell, has been shown in the intestine to occur through neutral competition of stem cell progeny for niche space, or neutral drift⁴⁰. In the adult antrum, each gland houses 3-4 active stem cells in its base²⁹. Gastric glands also exhibit monoclonality, with an entire gastric gland being populated by a single stem cell. This conclusion comes from studies by Nomura et al.⁴¹ where they followed expression of an X-linked LacZ transgene, which is inactivated randomly in females, to observe the blue

(LacZ-positive) white patterning to determine if the gland was being fed by one or multiple stem cell. In adult mice, most units were completely blue or white, suggesting monoclonality⁴¹. This finding was verified and expanded by the Barker lab with observation of LGR5-positive stem cells in the antrum drifting towards monoclonality over time⁴².

A key approach to determining if a specific population of cells are stem cells is lineage tracing. This technique marks a single cell with a permanent genetic reporter; thus all the cell's progeny will retain the mark resulting in labeled clones. Lineage tracing provides information about the number of progeny from a specific cell, their location, and the differentiated cell types that come from a specific cell⁴³. For gastric stem cells, a long-lived lineage trace is observed in an entire gastric gland and encompasses all differentiated cell types of either the antrum or corpus. The Clevers lab identified *Lgr5* as a marker of antral stem cells by observing full gland lineage tracing with a *Lgr5* gene-induced genetic mark that persisted for over a year²⁹.

Gastrointestinal organoids

Recently, there have been significant advances in the *in vitro* culture of mouse and human gastrointestinal epithelial cells. The intestinal organoid culture technique was pioneered by the Clevers' lab and involves the establishment of intestinal stem cells in culture to form physiologically relevant 3D spheroids that thrive without the support of mesenchymal cells, and has been applied to other tissues such as the stomach (Fig 1-4)⁴⁴. Intestinal organoids exhibit crypts with morphology similar to the adult small intestine though they lack villi. Cellular proliferation,

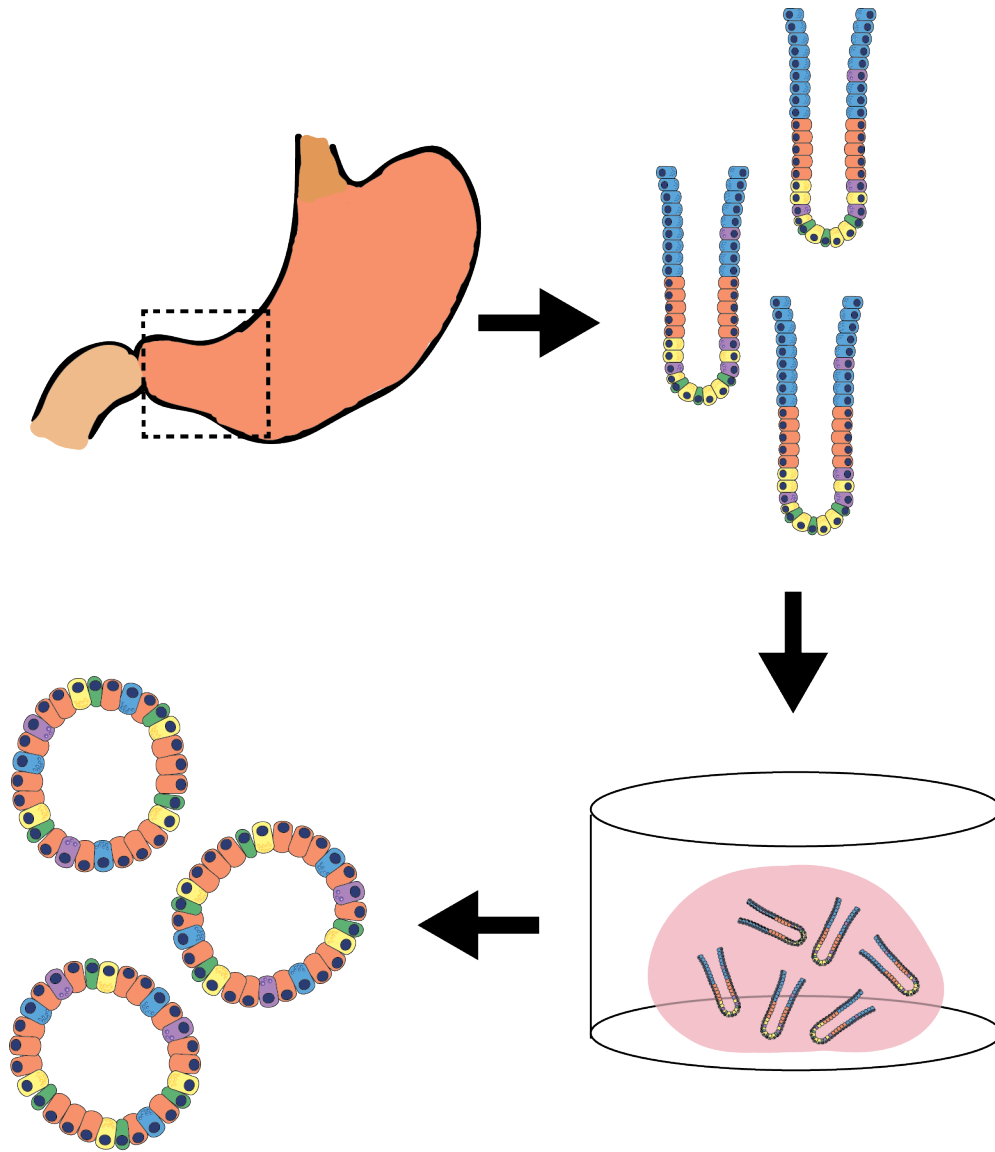


Figure 1-4. Schematic of gastric organoid establishment. For human and mouse gastric organoid establishment, glands are isolated from gastric tissue and plated in Matrigel. With the addition of specific growth factors, such as Wnt, R-spondin, and Noggin, spheroids contain differentiated cell types typical for the mouse stomach.

differentiation, and apoptosis in these organoids mimics *in vivo* cellular homeostasis⁴⁴. This system is a powerful tool due to the ability to sustain growth of intestinal stem cells *in vitro* for many months to years⁴⁴.

The development of organoids from single isolated cells has proven to be another key test to validate gastrointestinal stem cell identity. Single LGR5-expressing intestinal cells were demonstrated to form organoids *in vitro*, which present crypt-villus compartments and all differentiated cell types of the adult epithelium⁴⁴. *Lgr5*-expressing antral cells were also shown to form long-lived antral organoid cultures²⁹. The use of organoids is a powerful approach to test multipotency and stemness as well as epithelial-specific regulation of stem cell function.

Since the introduction of intestinal organoids by Sato et al⁴⁴, the technique has been adapted to other organs from the neonatal and adult mouse such as the liver⁴⁵, pancreas⁴⁶, and stomach²⁹, but also to human tissues from the stomach, small intestine, and colon^{47,48}. The organoid culture system has become an exciting new tool in the study of human development, disease, and homeostasis across numerous tissue types.

Markers of gastric stem cells

A significant limitation of our current understanding of gastric stem cell regulation is the few robust markers identified for both active and quiescent gastric stem cells. These markers are useful for visualizing stem cells and for generation of cell-specific genetic tools to study how a marked cell population might contribute to gastric epithelial homeostasis. Below are summaries of defined gastric stem or progenitor cell markers. Importantly most of these conclusions were defined in a single mouse study

and are generally not thoroughly characterized. A summary of gastric stem cell markers is provided in Table 1-1.

Similar to the intestine, the Wnt target gene *Lgr5* marks adult stem cells located at the base of the antral glands²⁹. There are 3-4 long-lived LGR5-positive stem cells located at the base of each gland that give rise to all differentiated cell types of the antral epithelium. *Lgr5*-expressing cells were found to be able to give rise to all cell types of the antrum with long-term renewal, with lineage tracing persisting over 620 days. Isolated LGR5-positive cells are also capable of generating and maintaining gastric organoids *in vitro* that give rise to antral cell types²⁹. LGR5-expressing cells are present throughout the gastric antrum, with few LGR5-expressing cells present in the corpus region, mainly confined to chief cells along the lesser curvature of the stomach⁴⁹. LGR5 also marks stem cell precursors in the corpus and antrum of immature mouse stomachs²⁹.

SOX2 marks uncommitted cells in the gastric corpus and antrum⁵⁰. Long-term lineage tracing from a *Sox2-CreERT2* transgenic mouse showed that *Sox2*-positive cells give rise to all differentiated cell types in both the corpus and antrum. SOX2 does not mark LGR5-positive cells. Long-term lineage tracing showed persistent tracing of both corpus and antral glands for over a year. Co-staining of SOX2 with the proliferation marker Ki67 showed that roughly half of the SOX2-positive cells are actively cycling. Analysis of *Sox2-CreERT2;ROSA26-IsI-eYFP* mice one day or one week after tamoxifen administration showed that some *Sox2*-positive cells are cycling and give rise to eYFP patches after one week, while other *Sox2*-positive cells appear to be slow cycling and remain as singly labeled cells⁵⁰.

Table 1-1. Description of genetic markers of mouse gastric stem cells.				
Gene	Mouse Model	Corpus or Antrum	Active or Quiescent	Reference
<i>Lgr5</i>	<i>Lgr5-EGFP-ires-CreERT2</i>	Antrum	Active	29
<i>Sox2</i>	<i>Sox2-CreERT2</i>	Both	Active	50
<i>Cckbr</i>	<i>CCK2R-CreERT-BAC</i>	Antrum	Active	51
<i>Lrig1</i>	<i>Lrig-CreERT2</i>	Both	Quiescent	36
<i>Vil1</i>	<i>Vil-LacZ</i>	Antrum	Quiescent	37
<i>Tnfrsf19</i>	<i>Troy-eGFP-ires-CreERT2</i>	Corpus	Quiescent	35

The gastrin receptor cholecystikinin B receptor (*Cckbr* also designated as *Cck2r*) labels long-lived actively cycling stem cells of the antrum⁵¹. Analysis of *Cck2r-CreERT-BAC* transgenic mice crossed to a ROSA reporter mouse identified CCK2R-expressing cells near the base of antral glands above LGR5-expressing cells. Tamoxifen activation of gave rise to long-lived lineage tracing of antral glands, including all mature antral cell lineages⁵¹.

LRIG1 has been suggested to be a quiescent intestinal stem cell marker based on slower proliferation rates than active LGR5-marked stem cells^{36,52}. Limited data has been published in regard to expression in gastric stem cells, but lineage-tracing studies showed long-lived tracing from a *Lrig-CreERT2* transgene in both the corpus and antral regions of the mouse stomach³⁶.

A rare subpopulation of gastric progenitors was identified by a marked allele of the villin (*Vil1*) gene, *Vil-LacZ*³⁷. These LacZ-positive cells were localized in the antrum at or below the isthmus region of the gland. This is a quiescent population that does not normally proliferate or contribute to normal gastric epithelial lineages. However, after stimulation with the proinflammatory cytokine interferon- γ this population exhibits multilineage potential with lineage-traced cells populating entire antral glands³⁷. These cells are not seen in the corpus.

Tnfrsf19 (*Troy*) is a marker of a subset of fully differentiated chief and parietal cells in the gastric corpus³⁵. A *Troy-eGFP-ires-CreERT2* knock-in mouse was generated and expression of eGFP was seen at the base of corpus glands in occasional chief and parietal cells. Crossing to a ROSA reporter strain showed rare lineage tracing of full corpus glands after four weeks that persisted for at least 1.5 years. Single *Troy*-positive

cells were shown to form gastric organoids *in vitro*. In a damage model where proliferating cells were killed by 5-fluoruracil (5-FU), *Troy*-positive cells showed accelerated expansion and the number of lineage tracing events increased 6-fold³⁵.

Tff2-CreERT2 BAC transgenic was found to mark progenitors for a subset of gastric cell types in the corpus⁵³. *Tff2-CreERT2* did not however mark a stem cell as lineage tracing was not observed in all cell types and the marks were not long-lived. *Tff2-CreERT2* expressing cells in the corpus marked progenitors of chief and parietal cells, but not surface mucous or enteroendocrine cells⁵³.

Mist1 is a transcription factor expressed in differentiated chief cells²¹. In a *Mist1-CreERT2* mouse model challenged with a spasmolytic polypeptide-expressing metaplasia (SPEM)-inducing drug, lineage tracing showed that the emerging TFF2-positive SPEM lineage traced from the *Mist1-CreERT2* cells. *Mist1*-positive cells have the capacity to act as progenitors to reacquire proliferative ability and give rise to SPEM cells⁵⁴, but not all cell types of the epithelium, indicating that *Mist1* is not a stem cell marker.

Pathways regulating gastric epithelial proliferation

Various signaling pathways have been shown to be key regulators of epithelial proliferation and/or differentiation in the stomach. Below are short summaries of some of the most well characterized signaling pathways and their role in the stomach.

Hedgehog signaling was identified as a contributor to gastric physiology after observation that Hedgehog ligand (sonic hedgehog (Shh)) null mouse mutants expressed metaplastic changes in the stomach⁵⁵. The specific role Shh plays in gastric

maturation was difficult to identify due to Shh-null mouse death within 24 hours of birth⁵⁵. In the normal adult mouse stomach, Shh expression is high in the forestomach and corpus and lower in the antrum, with the greatest expression in parietal cells^{56,57}. In adult tissue, analysis is limited due to the lack of stomach-specific promoters for Cre recombinase. To test Shh function in specific cell types, Xiao et al. used the H/K-ATPase driven constitutive Cre to delete Shh in parietal cells⁵⁸. These mice develop hypergastrinemia and an expanded surface mucous cell region⁵⁸. These studies suggest that Hedgehog signaling is important for maintenance of proper gastric physiology, but a specific regulatory role for gastric stem cells remains unknown.

There is little known about the role of Wnt signaling in normal gastric homeostasis. Wnt is a key factor in maintenance of *in vitro* gastric organoid cultures and *Lgr5*, a marker of antral stem cells, is a Wnt target gene, suggesting that Wnt signaling is important for stem cell maintenance²⁹. Deletion of the Wnt negative regulators *APC* or *GSK3* in the stomach led to rapid loss of parietal cells and fundic gland polyp formation⁵⁹. Deletion of *APC* from antral stem cells using *Lgr5-EGFP-CreERT2* resulted in gastric adenoma formation²⁹. These studies suggested that Wnt signaling is key to maintaining normal gastric homeostasis and further analysis will be necessary to detail Wnt function in the stomach.

Various receptors and ligands of BMP signaling are expressed in both epithelial and mesenchymal cells of the stomach, including BMP2 in epithelial cells and BMP4 in non-epithelial myofibroblast-like cells⁶⁰. Loss of BMP signaling in early development impacts overall stomach patterning, with an expansion of the foregut region⁶¹. Expression of the BMP antagonist noggin in parietal cells in the adult mouse resulted in

a loss of parietal cells, the development of transitional cells that express both mucus neck and zymogenic lineages, and activation of proliferation²⁰. These profound abnormalities in maturation and differentiation indicate that BMP signaling plays a crucial role in the regulation of normal gastric homeostasis.

TGF α is produced by the gastric mucosa and inhibits acid secretion, stimulates cell migration and proliferation after injury, and regulates gastric mucus levels⁶². TGF α has been implicated in the pathogenesis of Menetrier's disease, a disorder of the stomach characterized by corpus gland hyperplasia⁶³.

Gastrin is a key hormonal inducer of acid secretion in the stomach. Gastrin also serves as a growth factor by stimulating epithelial proliferation and a thickening of the gastric mucosa⁶⁴. It has been suggested that the growth-promoting effect of gastrin in the corpus may be due to direct interaction of gastrin with progenitor cells, but this remains debatable^{51,65}.

1.2: THE NOTCH SIGNALING PATHWAY

Notch signaling is an evolutionarily conserved pathway that plays a critical role in multiple developmental programs at various stages. Notch signaling links the fate of neighboring cells, with physical interaction of membrane-bound Notch receptors with a ligand expressed in an adjoining cell (Fig 1-5). The outcome of Notch signaling is dependent on the cellular context, and can include proliferation, terminal differentiation, and lineage commitment. Examples of the variety of outcomes offered in various mammalian tissues have been summarized in Table 1-2. The Notch signaling pathway

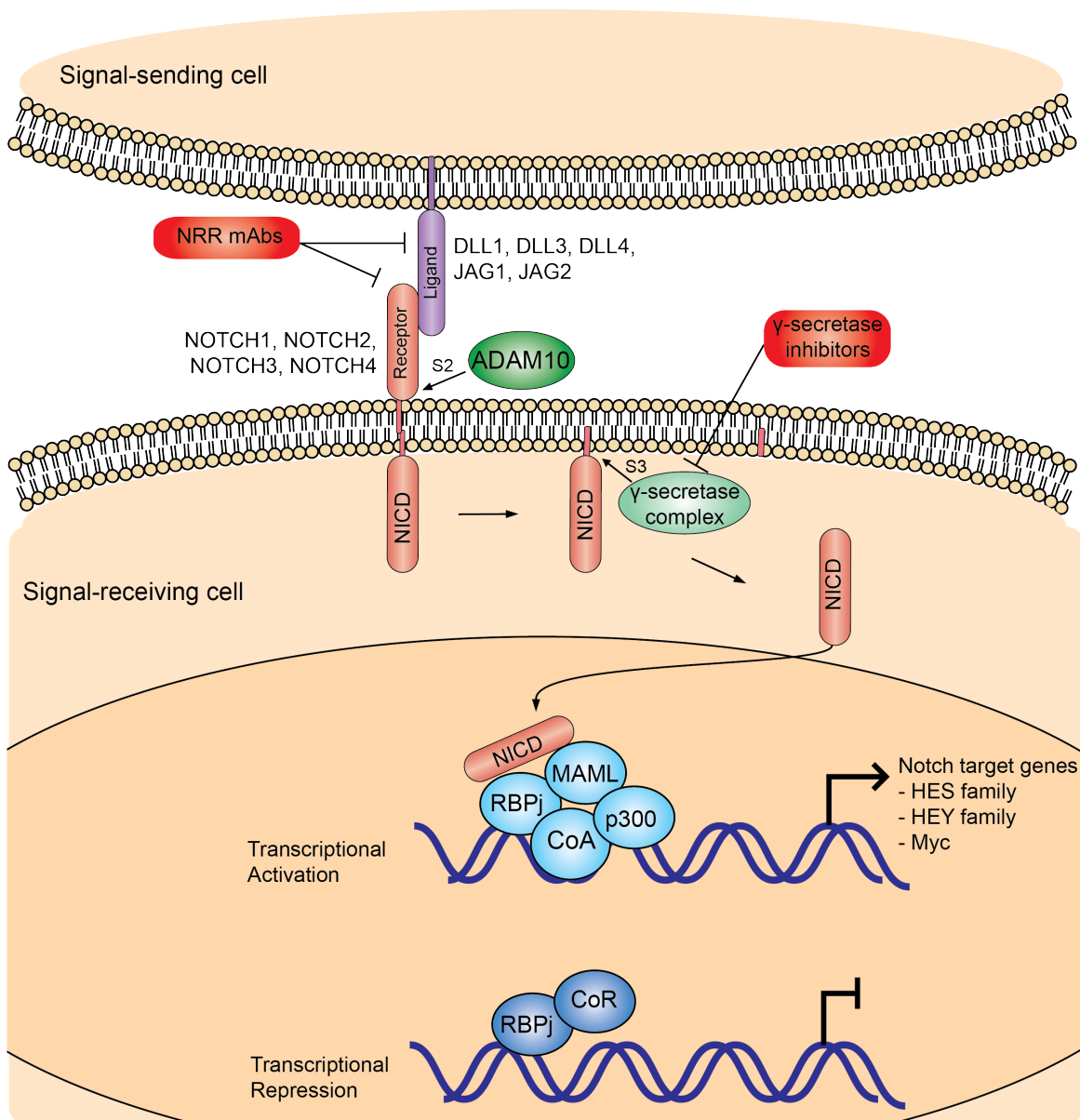


Figure 1-5. Schematic of the Notch signaling pathway. Upon binding of a Notch ligand with a Notch receptor, the negative regulatory region of the Notch receptor undergoes a conformational change and exposes a cleavage site. ADAM10 cleaves the Notch extracellular domain from the Notch intracellular domain (NICD). A γ -secretase complex cleaves the NICD from the transmembrane domain to release it for translocation to the nucleus. Binding of NICD to the DNA-binding protein RBPJ releases co-repressors (CoR) and allows for association with co-activators (CoA, mastermind-like (MAML), p300) to lead to Notch target gene transcription.

Table 1-2. Role of the Notch signaling pathway in various organs/tissues.

Organ/Tissue	Processes Regulated
Brain	Balance of gliogenesis and neurogenesis, stem cell maintenance, apicobasal polarity ^{66,67}
Breast	Alveolar development, maintenance of luminal cell fate, regulation of basal cell proliferation ⁶⁸
Craniofacial structures	Palate morphogenesis, tooth development ⁶⁹⁻⁷³
Ear	Defines sensory epithelium, hair and supporting cell fate decision ⁷⁴⁻⁷⁶
Esophagus	Regulates epithelial homeostasis ⁷⁷
Eye	Fiber cell differentiation in lens development ⁷⁸⁻⁸⁰
Heart	Cardiac patterning, cardiomyocyte differentiation, valve development, ventricular trabeculation, outflow tract development ⁸¹
Hematopoietic system	Balances B-cell versus T-cell development, maintenance of stem cells, maintenance of myeloid homeostasis ⁸²
Intestine	Controls proliferation and differentiation, stem cell maintenance ^{2,6,83}
Kidney	Cell fate of podocytes and proximal tubules ⁸⁴
Limbs	Apical ectodermal ridge formation and digit morphogenesis ^{69,85-88}
Liver	Ductal plate formation, intrahepatic bile duct morphogenesis ^{71,89-91}
Lungs	Lateral inhibition between tracheal cells ⁹²
Muscle	Promotes transition of satellite cells to myogenic precursor cells and myoblasts ⁹³
Neural crest	Controls patterning of neural crest precursors, regulates transition of Schwann cell precursor to Schwann cell, controls Schwann cell proliferation and inhibits myelination, melanocyte stem cell maintenance ⁹⁴⁻⁹⁶
Pancreas	Directs endocrine cell differentiation, maintain endocrine precursor cells, inhibits terminal acinar cell differentiation, controls epithelial branching and bud size ⁹⁷
Pituitary	Regulates pituitary growth/proliferation, melanotrope specification and gonadotrope differentiation ⁹⁸⁻¹⁰⁰
Placenta	Controls fetal angiogenesis, maternal circulatory system development, spongiotrophoblast development ¹⁰¹
Prostate	Epithelial differentiation and growth ¹⁰²⁻¹⁰⁴
Sex organs and germ	Maintenance of Leydig progenitor cells, regulation of

cells	spermatogenesis, controls oocyte growth ¹⁰⁵⁻¹⁰⁸
Skin	Regulates cell adhesion, controls proliferation, controls hair follicle or feather papillae differentiation and homeostasis ^{109,110}
Spine	Somite segmentation ^{111,112}
Spleen	Regulates generation of T lineage-restricted progenitors and marginal zone B-cell development, controls homeostasis of dendritic cells ¹¹³
Stomach	Regulation of epithelial proliferation in corpus and antrum, determine cell fate choice in antrum, regulates stem cells in the antrum, regulates cell fission ^{10,11,114}
Thymus	Thymic morphogenesis, differentiation of gamma delta lineage T-cells ⁶⁹
Thyroid	Regulates the number of thyrocyte and C-cell progenitors and regulates differentiation and endocrine function of thyrocytes and C-cells ¹¹⁵
Vasculature	Regulates arteriovenous specification and differentiation in endothelial cells and smooth muscle cells, regulates blood vessel sprouting and branching ¹¹⁶

is important in the regulation of adult stem cells in the gut, skin, muscle, hematopoietic system, and lungs¹¹⁷. The Notch pathway must be able to produce spatially and temporally appropriate responses, as Notch malfunction has been linked to diseases with altered cellular proliferation, such as cancer.

Notch receptors and ligands

In canonical Notch signaling, transmembrane Notch receptors interact extracellularly with transmembrane Notch ligands on an adjacent cell. This initiates proteolytic cleavage of the Notch receptor to release the Notch receptor intracellular domain (NICD). There are four Notch receptors (NOTCH1-4) that are multidomain proteins with structures that have been conserved from invertebrates to humans¹¹⁸. There are five Notch ligands, three from the Delta-like family (DLL1, DLL3, DLL4) and two from the Jagged family of Serrate homologs (JAG1, JAG2)¹¹⁹. This allows for a significant number of unique receptor-ligand combinations, but there is little evidence for distinct responses from particular combinations. Instead, tissue and cell specific expression of receptors and ligands appears to account for the varying responses to Notch signaling.

Structure of Notch receptors and ligands

The Notch receptor is composed of an extracellular domain (NECD) and intracellular domain (NICD) tethered by a transmembrane domain that can be cleaved to release NICD (Fig 1-6). NECD consists of 29 to 36 EGF homology repeats, which vary for different Notch receptors. These EGF repeats mediate interaction with ligands and can be modified post translationally to alter Notch function¹¹⁸. NECD is followed by

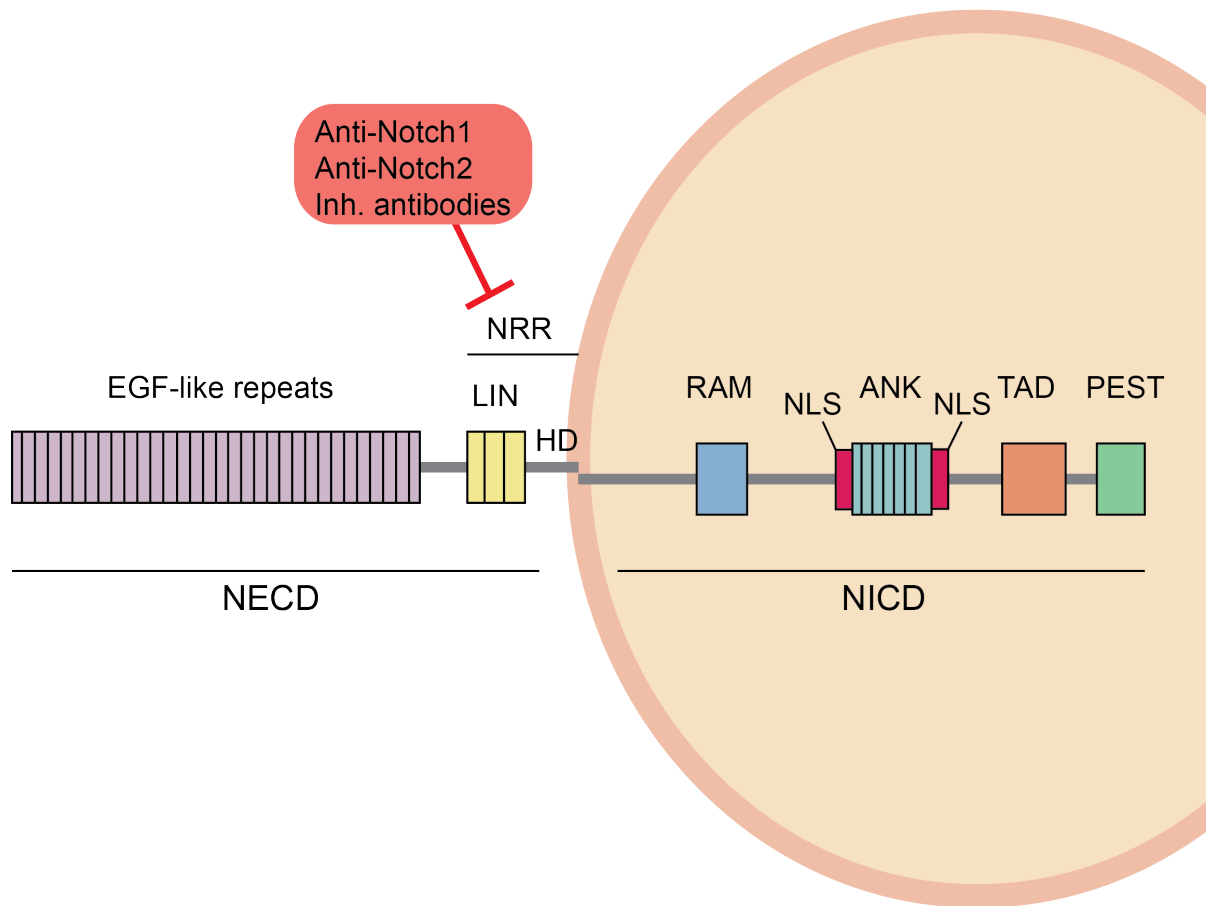


Figure 1-6. Schematic of the Notch receptor structure. The extracellular region of the Notch receptor (NECD) is composed of 29 to 36 epidermal growth factor (EGF) repeats and the negative regulatory region (NRR), which is composed of three cysteine-rich Lin-12-Notch repeats (LIN) and a heterodimerization domain (HD). The Notch receptor intracellular domain (NICD) is composed of the RBPJk associated module (RAM), two nuclear localization sequences (NLS) flanking seven anykrin repeats (ANK), the transcriptional activator domain (TAD) and a C-terminal proline, glutamic acid, serine, threonine-rich (PEST) domain. Inhibitory antibodies can block Notch signaling by targeting the NRR of specific receptors.

the negative regulatory region (NRR), which prevents access to the S2 cleavage site in the absence of Notch ligands. The NRR is composed of three Lin-12-Notch repeats (LIN) and a hydrophobic region for receptor heterodimerization (HD)^{120,121}. Following the transmembrane domain, NICD is composed of the RBPJk association module (RAM), two nuclear localization sequences (NLS) flanking a seven ankyrin repeats (ANK) domain, a transcriptional activation domain (TAD) and a C-terminal proline/glutamic acid/serine/threonine-rich (PEST) motif¹²¹. RAM and ANK are essential to allow NICD to target RBPJ in the nucleus, and PEST is a target for NICD degradation^{121,122}. The Notch ligands are transmembrane proteins that have an extracellular domain with EGF-like repeats and a N-terminal Delta/Serrate/lag-2 (DSL) domain, which is required for interaction with Notch receptors¹²².

Notch receptor processing

Release of NICD requires multiple cleavage events of the Notch receptors. The first cleavage event, S1, is a Furin-dependent cleavage that occurs in NECD during trafficking through the Golgi complex¹²³. The NRR plays a crucial role in preventing subsequent S2 cleavage from occurring in the absence of ligand binding by conformational blocking of the cleavage site. Many studies have shown NRR to be the “activation switch” of the receptor through the discovery that receptors that lack the EGF-like repeats are functionally inert^{124–126} and deletion or point mutation of the LIN module lead to gain-of-function phenotypes¹²⁷. Binding of a Notch ligand to a Notch receptor induces a conformational change that relaxes and unfolds the NRR, exposing the S2 cleavage site¹²⁸. S2 cleavage event is performed by an ADAM metalloprotease,

with multiple ADAM family members being implicated in Notch receptor cleavage events^{129–133}. However, more recent *in vivo* data has definitively shown that ADAM10 is responsible for S2 cleavage¹³³. ADAM10 cleavage generates the activated intermediate termed the Notch Extracellular Truncation (NEXT)^{129,134}. NECD is trans-endocytosed into the signal-sending cell and can be recycled back to the plasma membrane or sorted into late endosomes¹³⁵. The tethered NEXT is then the site for S3 cleavage, which is completed by the γ -secretase complex that contains Presenilin¹³⁶. After S3 cleavage the NICD is released from the membrane and is free to translocate to the nucleus.

Notch intracellular domain

Upon ligand binding and proteolytic processing of the Notch receptor, NICD is able to translocate directly to the nucleus. In the nucleus NICD forms a transcriptional complex with the DNA binding protein RBPJk through the RAM domain, Mastermind (MAML), and various transcriptional co-activators to activate expression of Notch target genes^{120,121}. In the absence of NICD, RBPJk forms a complex with co-repressors and histone deacetylases to prevent activation of Notch target genes^{120,121}. NICD in the nucleus is stabilized through phosphorylation by kinases such as glycogen synthase kinase 3 β (GSK3 β) and cyclin-dependent kinase 8 (CDK8)^{137–139}. NICD is targeted for proteasomal degradation by the E3 ubiquitin ligase SEL10^{140,141}. The Notch signaling pathway lacks amplification steps, with each NICD being consumed during signaling.

Notch target genes

Notch signaling in different cell types reveals considerable variety in the immediate downstream Notch response. The best characterized Notch target genes belong to the hairy and enhancer of split-related (HESR) family that code for basic helix-loop-helix (bHLH) transcription factors. HESR genes play an important role in promoting cell fates by repressing genes such as atonal homolog 1 (*Atoh1*), achaete-scute like 1 (*Ascl1*) and Neurogenin families of bHLH transcriptional activators^{142,143}. *C-myc*, *CyclinD*, *CDK5*, and *CDC25* have been shown to be Notch target genes in cells where Notch promotes proliferation^{144–147}.

Notch in adult stem cell homeostasis

Notch signaling has been well characterized as a key mediator of stem cell homeostasis across numerous tissues. Below are short summaries of the role of Notch signaling in maintenance of skin, muscle, hematopoietic, and lung stem cells.

The skin epidermis is a rapidly renewing epithelium that serves as a barrier from environmental factors and to prevent dehydration. The skin epidermis is comprised of four layers: basal, spinous, granular, and cornified, with basal cells capable of self-renewal and production of new cells to differentiate and move outwards¹⁴⁸. RBPJ is expressed throughout all four layers, but the Notch receptors NOTCH1, NOTCH2, and NOTCH3 are only present in the spinous layer¹⁴⁹. A mouse genetic model of Notch inhibition through *Rbpj* deletion resulted in an absence of spinous cells due to a lack of cell exit from the niche and differentiation from the basal layer¹⁵⁰. Interestingly, the loss of Notch signaling triggered hyperproliferation via cytokine production and led to a tumor-promoting environment, leading some to believe that Notch has a “tumor

suppressor” phenotype in the skin¹⁵¹. The Notch signaling pathway does not play a role in maintenance of quiescent stem cells in the skin¹¹⁸.

Adult muscle growth and repair is dependent on the specialized subset of muscle cells, the satellite cells¹⁵². Satellite cells are thought to be a mix of stem cells and more committed progenitor cells. Normally in a quiescent state, satellite cells can be activated upon damage to the muscle fibers to fuse and make myofibers that regenerate the tissue¹⁴⁸. Various Notch components are expressed in satellite cells, such as DLL1, JAG1, NOTCH1, NOTCH2, NOTCH3 and Notch target genes such as *Hes1* and *Hey1*^{153,154}. Constitutive expression of Notch1 or the overexpression of DLL1 in cultured myogenic cells inhibited myogenic differentiation^{155–157}. Conversely, inhibition of the Notch signaling pathway through overexpression of the negative regulator Numb or administration of γ -secretase inhibitors promotes myoblast differentiation and stimulates formation of larger myotubes^{155,156,158}. These data indicate that Notch plays a key role in promoting proliferation of satellite cells while inhibiting terminal differentiation.

Hematopoietic stem cells in bone marrow give rise to myeloid and lymphoid precursors that differentiate to form red and white blood cells (T cells, B cells), megakaryocytes, and other cell types¹⁴⁸. Notch signaling is crucial for the generation of adult hematopoietic stem cells, with NOTCH1 and JAG1 required for development of differentiated cells^{159,160}. The role for adult stem cell homeostasis is unclear. The overexpression of Notch components results in the increased renewal of hematopoietic progenitors¹⁶¹. However, knock out of Notch components such as NOTCH1 and JAG1 have no effect on hematopoietic stem cell homeostasis¹⁶². It is known that Notch is essential for the maturation of the T cell lineage. Loss of Notch signaling by genetic

deletion of NOTCH1 or RBPJ results in a loss of T cells and an increase in B cells, showing Notch promotion of a binary cell fate decision between these two cell types^{162,163}. Notch is important for lineage commitment and terminal differentiation, but its role in maintenance of hematopoietic stem cells remains unclear.

The mammalian respiratory system consists of branched tubular structures that enable functional gas exchange. The lung epithelium contains undifferentiated basal cells, the stem cells of the lung epithelium, that give rise to differentiated ciliated and secretory cells¹⁶⁴. There is very little turnover of the lung epithelium in steady-state; self-renewal only occurs after damage to the tissue¹⁶⁴. At steady-state, NOTCH1, DLL1, JAG1, and JAG2 are expressed in the basal cells and, upon damage, expression is increased along with expression of Notch target genes¹⁶⁵. The expression of NICD in the lungs promoted differentiation of the secretory Clara cells, but not neuroendocrine cells, indicating Notch promotion of the secretory cell fate¹⁶⁵. This indicates that Notch is involved in directing cell fate choices in certain differentiated cell types of the lung epithelium.

Notch in disease

The Notch signaling pathway plays a key role in stem cell maintenance in numerous tissues throughout mammals. However, mutations in various Notch components do occur and can lead to a number of diseases in humans. Table 1-3 summarizes disorders associated with mutations of Notch pathway components.

Table 1-3. Known Notch mutations associated with human diseases^{118,122,166,167}

Disease	Symptoms	Notch Genes Associated
Alagile Syndrome	Developmental abnormalities in heart, liver, eye, and skeleton	<i>JAG1, NOTCH2</i>
Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) Syndrome	Autosomal vascular disorders linked to ischemic strokes, dementia, and premature death	<i>NOTCH3</i>
T cell acute lymphoblastic leukemia (T-ALL)	Anemia and enlargement of lymph nodes in liver or spleen	<i>NOTCH1</i>
Spondylocostal dysostosis	Vertebral segmentation defects, rib anomalies	<i>DLL4</i>
Hajdu-Cheney Syndrome	Bone resorption leading to osteoporosis	<i>NOTCH2</i>

1.3: REGULATION OF GASTROINTESTINAL STEM CELLS BY NOTCH

Notch intestinal function

The small intestine is similar to the gastric antrum, with LGR5-positive stem cells at the base of intestinal crypts giving rise to all differentiated cell types of the intestinal epithelium. Notch signaling affects both stem and differentiated cell types. DLL1 and DLL4 are the key Notch ligands and NOTCH1 and NOTCH2 are the key Notch receptors for intestinal cell regulation^{4,5,8}. Notch promotes intestinal stem cell proliferation and directs progenitor cells to the absorptive cell fate by inhibiting secretory cell differentiation. Loss of Notch signaling in the intestine through pharmacological inhibition of γ -secretase, or genetic knock-out of RBPJk or NOTCH1/NOTCH2 resulted in loss of epithelial proliferation and secretory cell hyperplasia^{2,3,6,8,168,169}. Conversely, genetic activation of the Notch signaling pathway by overexpression of NICD was reported to increase epithelial proliferation and inhibit secretory cell differentiation⁸³.

The secretory cell fate inhibition by Notch occurs through negative regulation of the transcription factor atonal homolog 1 (*Atoh1*). Genetic mouse models have shown that constitutive *Atoh1* expression promotes secretory cell differentiation, and *Atoh1* gene deletion leads to the loss of the secretory cell lineage (goblet, endocrine, and Paneth cells)^{170,171}.

Notch inhibition by γ -secretase inhibitors causes a downregulation of the intestinal stem cell marker olfactomedin 4 (*Olfm4*) in the intestine and transcription studies identified *Olfm4* as a direct Notch target gene³. *Olfm4* has been shown to be a specific intestinal stem cell marker¹⁷². The observation that Notch signaling directly

targets *Olfm4* expression demonstrates that Notch signaling is in the intestinal stem cell³.

Notch gastric function

Compared to intestinal stem cells, the role of Notch in maintenance of gastric stem and epithelial cell homeostasis has not been well characterized. Kim et al. first outlined the role of Notch signaling in both the developing and adult stomach with findings described below¹⁰. Utilizing *in situ* hybridization, they showed multiple Notch ligands and receptors were expressed throughout the mouse embryonic epithelium, including *Notch1*, *Jag2*, and *Dll3*, as well as the Notch target gene *Hes1*. After administration of a γ -secretase inhibitor to block Notch signaling in adult mice, they observed a marked reduction of epithelial proliferation in both the corpus and antrum, which was accompanied by a significant increase in both mucous and enteroendocrine cells in the antrum. To investigate the consequence of Notch over-activation, a genetic mouse model was used that constitutively expressed NICD in parietal cells. These Notch-activated parietal cells dedifferentiated and had stem-like characteristics, giving give rise to glands containing all cell types of the corpus epithelium. Over time, the Notch-activated corpus formed hyperproliferative adenomas with focal activation of the Wnt pathway. However, in this study, when NICD was constitutively expressed in LGR5 cells in the antrum, proliferation was not affected and adenomas did not develop. From these findings Kim concluded that Notch signaling is present in both the developing and the adult stomach and is required for maintaining gastric epithelial homeostasis¹⁰.

Demitrack et al. established an important role for Notch signaling to regulate LGR5 antral stem cells¹¹. Using pharmacological Notch inhibition through a γ -secretase inhibitor, proliferation of LGR5-positive stem cells was decreased. Using the same genetic model of Notch activation as the Kim study to drive NICD expression in Lgr5-positive cells, they observed increased antral epithelial and stem cell proliferation, contrary to the previous study. The NICD constitutive Notch activation model exhibited an increase in gland fission events and a rapid generation of monoclonal glands, indicating an increase in the number of stem cells per gland. Importantly, chronic Notch activation induced undifferentiated, hyper-proliferative polyps, suggesting that Notch hyper-activation in gastric stem cells may contribute to gastric tumorigenesis¹¹.

Analysis of differentiation in the Demitrack study showed that Notch inhibition increased all differentiated cell types of the antrum, including surface and deep mucous cells and enteroendocrine cells. Conversely, genetic Notch activation led to a decrease in all differentiated cell types¹¹.

These studies have highlighted the importance of Notch signaling in maintaining gastric epithelial homeostasis and the potential for Notch signaling to contribute to gastric cancer development. However, key questions remain in how Notch achieves this regulation. 1) What is the mechanism through which Notch regulates gastric epithelial homeostasis and 2) which pathway components are involved? These are the questions investigated in this thesis.

Expression of Notch receptors and ligands in gut

The roles of individual Notch receptors and ligands are starting to be characterized. The adult intestine expresses *Notch1*, *Notch2*, and *Notch3* receptors and *Dll1*, *Dll4*, *Jag1*, and *Jag2* ligands^{173,174}. *Notch1*, *Jag1*, and *Jag2* were expressed in the lower third of all crypts, in the proliferative region, while *Notch2* was expressed in rare cells in select crypts¹⁷³. *Notch1* was also abundantly expressed in the lamina propria, and both *Notch1* and *Notch2* showed expression in the underlying muscle layers¹⁷³. *Notch3* was expressed primarily in the vasculature¹⁷⁴. To determine which cells express the Notch receptors, a set of knock-in mice was developed in which the Cre-estrogen receptor binding domain fusion was knocked-in to the first exon of each of the four Notch receptors (Notch1-4) and crossed to a floxed ROSA reporter strain⁴. Notch3 and Notch4 were not expressed in the intestinal epithelium, but Notch1 and Notch2 gave rise to long-lived labeled intestinal clones suggesting that these two receptors are expressed in intestinal stem cells⁴. The Notch1-Cre construct induced far greater numbers of lineage stripes compared to the Notch2-Cre expressing stem cells⁴.

Less is known about Notch receptor and ligand expression in the stomach. Northern blot analysis show gene expression of *Notch1*, *Notch2*, *Notch3*, *Jag1*, *Jag2*, and *Dll1* in both the forestomach and corpus region of the rat stomach¹⁷³. Analysis of the fetal mouse stomach showed expression of *Notch1*, *Jag2*, and *Dll3* by *in situ* hybridization in the epithelium at embryonic day 17¹⁰. My studies aimed to fill this gap by investigating the localization and function of the Notch receptors in the stomach.

Notch receptors and ligands in intestinal epithelial homeostasis

There have been various reports as to the role of the Notch receptors in maintaining intestinal epithelial homeostasis. Early studies indicated that NOTCH1 and NOTCH2 function redundantly, and only simultaneous loss of both receptors resulted in reduced epithelial proliferation and conversion of crypt progenitors into post-mitotic goblet cells, mimicking what is seen through γ -secretase inhibition⁶. The development of specific inhibitor antibodies, detailed below, against NOTCH1 or NOTCH2 expanded this finding to show that NOTCH1 plays a more dominant role in epithelial homeostasis^{9,175}. Inhibition of NOTCH1 alone was sufficient to induce goblet cell metaplasia; however, the effect was mild compared to inhibition of both NOTCH1 and NOTCH2. In contrast, inhibition of NOTCH2 alone did not induce any proliferative or goblet cell phenotype. This indicates that NOTCH1 and NOTCH2 function redundantly in intestinal cell differentiation, although inhibition of NOTCH1 is sufficient to reveal a partial phenotype^{9,175}. Similar results were observed with a genetic model of *Notch1* and/or *Notch2* deletion. In addition impaired crypt regeneration after radiation was observed in both *Notch1*-deleted and *Notch2*-deleted intestines, suggesting a need for higher Notch activity post-injury⁸. Also, gene expression profiling of isolated LGR5 intestinal stem cells demonstrated *Notch1* expression¹⁷⁶. Combined, these findings suggest that NOTCH1 is the primary receptor regulating intestinal stem cell homeostasis, and that both NOTCH1 and NOTCH2 regulate epithelial cell proliferation and differentiation in the adult intestine.

Investigation of the Notch ligands in the small intestine used genetic mouse models to delete *Dll1*, *Dll4*, and/or *Jag1*⁵. Deletion of *Jag1* or *Dll4* did not alter intestinal epithelial homeostasis, but deletion of *Dll1* did result in increased numbers of goblet

cells without effect on progenitor proliferation⁵. However, deletion of both *Dll1* and *Dll4* led to loss of epithelial proliferation and conversion of progenitors into goblet cells, mimicking global Notch inhibition phenotype. This indicates that DLL1 and DLL4 are required for the maintenance of intestinal progenitors and stem cells⁵. Paneth cells express DLL4 and a subset of transit-amplifying cells have been shown to express DLL1^{176,177}. Thus, DLL1 and DLL4 are the key Notch ligands in the intestinal stem/progenitor cells for epithelial and stem cell homeostasis.

Inhibition of Notch receptors by specific antibodies

The four Notch receptors are expressed in various mammalian tissues to direct cell fate decisions and growth. Although γ -secretase inhibitors have progressed as potential therapeutics for Notch-related pathologies, they fail to distinguish between individual Notch receptors and have targets other than the Notch-signaling pathway. In order to elucidate independent functions of discrete Notch receptors, Wu *et al.* developed inhibitory antibodies directed against the NRR of either NOTCH1 or NOTCH2¹⁷⁵. Utilizing phage display to generate antibodies targeting the NRR sequence, they showed that the antibodies potently inhibit NOTCH1 or NOTCH2 of either the human or mouse orthologue. Both antibodies inhibited signaling induced through different Notch ligands, including JAG1, JAG2, DLL1, and DLL4, indicating that inhibition occurs irrespective of the ligand. Examination of T cells and splenic marginal zone B (MZB) cell development proved functionality of specific receptor inhibition, because these processes depend specifically on Notch1 or Notch2, respectively. Treating mice with anti-Notch1 antibody specifically decreased T cells, while anti-

Notch2 decreased MZB cells, demonstrating the paralogue-specific inhibition¹⁷⁵. One main goal of the development of these antibodies was to circumvent the intestinal toxicity associated with γ -secretase inhibitors. Accordingly, administration of the Notch1-inhibitory antibody produced a partial goblet cell hyperplasia without fatal intestinal toxicity seen with γ -secretase inhibitors, representing a breakthrough over existing pan-Notch inhibitors for cancer therapeutics¹⁷⁵. I utilized these antibodies to study receptor function in the gastric epithelium.

Mouse models of Notch reporters

Multiple tools have been developed to better understand the role of Notch signaling and the components involved in various tissues. Below are descriptions of genetically engineered mice used in this study to better understand the role of Notch signaling in the mouse stomach. A summary of the mouse models and schematic of the genetic constructs are present in Table 1-4 and Fig 1-7.

The Hadjantonakais lab created a unique Notch signaling reporter mouse model that utilizes a CBF1 (also known as RBPJ κ) DNA binding site to provide single-cell resolution visualization of cells undergoing Notch signaling¹⁷⁸. This construct placed CBF1 binding sites into a promoter driving a human histone H2B nuclear localization sequence linked to the yellow fluorescent protein Venus to generate a transgenic mouse strain (CBF:H2B-Venus). This strain faithfully recapitulates Notch signaling in embryonic and adult tissue and is the first strain in which individual cells transducing a Notch signal can be easily visualized¹⁷⁸. This strain was used in Chapters 2 and 3 to identify cells in the adult mouse stomach that were undergoing Notch signaling.

Table 1-4. Mouse models to detect Notch signaling and receptor expression			
Mouse Model	Reports	Constitutive or Inducible	Reference
<i>CBF:H2B-Venus</i>	Active Notch signaling	Constitutive	178
<i>Notch1-CreERT2</i> and <i>Notch2CreERT2</i>	Notch1 or Notch2 receptor expression	Inducible	4
<i>Nip1-CreERT</i>	Active Notch1 signaling	Inducible	179

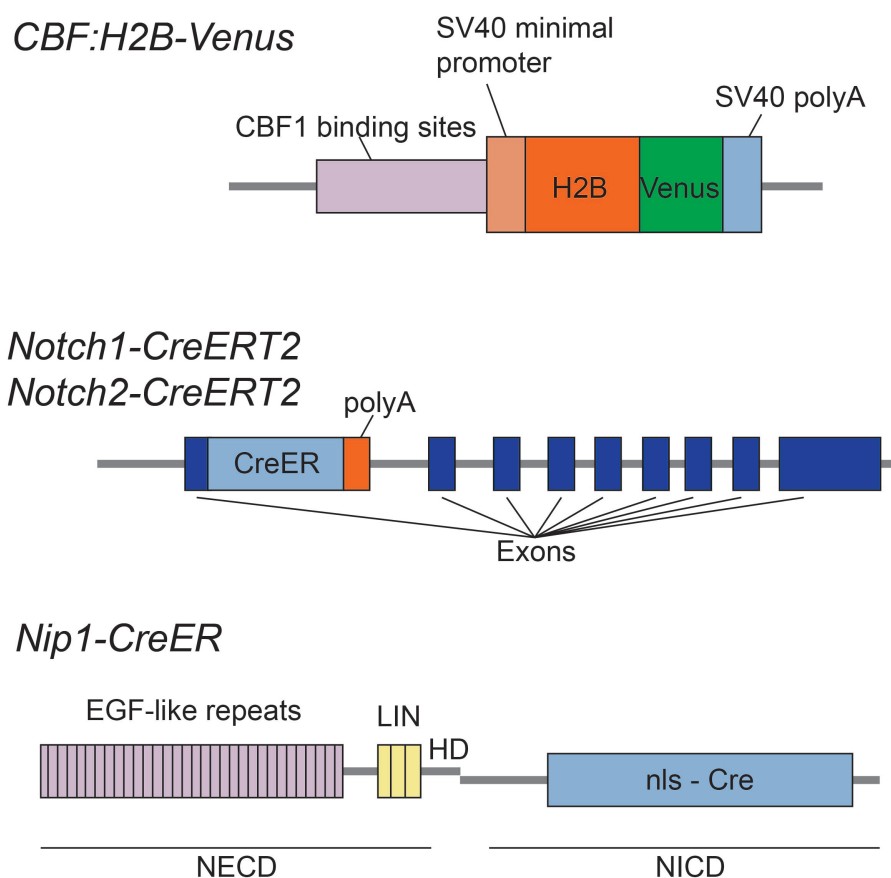


Figure 1-7. Schematic of genetic constructs for mouse models of Notch signaling and receptor expression. Mouse models described in text and Table 1-4.

As mentioned previously, the Artavanis-Tsakonas lab created a set of knock-in mice to identify cells that express the Notch receptors⁴. Transgenic mice were created by knocking-in the Cre-estrogen receptor binding domain fusion (Cre-ER) to the first exon of each of the four Notch receptors. When crossed to a transgenic reporter strain (such as *ROSA-LacZ* or *ROSA-Tomato*) and activating the Cre-ER by tamoxifen treatment, the expression pattern of each of the four Notch receptors can be observed. Longer chase periods after Cre-ER activation can allow for identification of stem cells expressing Notch receptors through lineage tracing. These transgenic lines can be used for genetic recombination in specific Notch receptor-expressing cells by crossing the mice to a transgenic line with a gene of interest flanked by lox-P sites. This set of knock-in mice identified *Notch1* and *Notch2* expression in stem cells of the intestine, as described above⁴, with *Notch1* having a more robust number of lineage tracing events compared to *Notch2*. I used these strains in Chapters 2 and 3 to map the expression of the Notch1 and Notch2 receptors in the adult mouse stomach.

The Kopan lab created a mouse model to detect active Notch signaling through the *Notch1* receptor¹⁷⁹. The genetic mouse model, named *NIP1-CreERT*, was engineered to replace NICD in the *Notch1* receptor with an inducible Cre recombinase. This results in Cre activity being governed by the ligand-induced proteolysis of Notch1. By crossing to a reporter strain, such as *ROSA-Tomato* and tamoxifen activation of the Cre, both expression and activity of *Notch1* can be visualized¹⁷⁹. This model was used by the Samuelson laboratory to identify *Notch1* expression and function in antral stem cells¹¹.

Notch receptors and ligands in human gastric cancer

There is evidence that dysregulation of the Notch signaling pathway can contribute to gastric cancer progression. Gastric cancer is the second leading cause of cancer deaths worldwide and 5-year survival rates are less than 40%¹⁸⁰. There are two types of gastric cancer based on histology: intestinal and diffuse. Intestinal type is characterized by gland-like tubular structures formed by cohesive neoplastic cells. Diffuse type is characterized by a poorly differentiated tumor that infiltrates the gastric wall. The cells of origin have not been determined, but gastric stem cells are strong candidates due to their high-proliferative potential and, as a regulator of gastric stem cell homeostasis, the Notch pathway may play a role in cancer initiation¹⁸¹.

Numerous studies have identified a correlation between human gastric cancer samples and Notch signaling, which are summarized in Table 1-5. One study found that levels of *Jag1* correlated with aggressiveness of human gastric cancer and that overexpression of NOTCH1 NICD promoted the colony-forming ability of a human stomach adenocarcinoma cell line¹⁸². Another study found that *NOTCH1* was rarely expressed in normal human gastric mucosa and was highly upregulated in premalignant cancer tissues, and that *NOTCH2* was detected in all four tested gastric cancer cell lines¹⁸³. *NOTCH1*, *NOTCH3*, *JAG1*, and *JAG2* expression was found to be increased significantly in tumor compared to normal tissue in another study, with *NOTCH3* and *JAG2* associated with intestinal type gastric cancer¹⁸⁴. In a different study the expression of *NOTCH1*, *NOTCH2*, *DLL4*, and *HES1* was significantly higher in tumor tissues compared to normal, with *NOTCH1* and *JAG1* expression correlated to poor prognosis¹⁸⁵. Together these studies, although each limited in scope, suggest that

Table 1-5. Notch signaling components associated with gastric cancer

Gene	Role in gastric cancer
<i>DLL4</i>	Expression of <i>DLL4</i> in human gastric cancer cell line promoted proliferation, migration, and invasion ¹⁸⁶
<i>NOTCH1</i>	Expression increased in gastric tumors compared to normal ¹⁸⁴ Expression of <i>NOTCH1-NICD</i> promoted colony forming ability and xenograft tumor growth of stomach gastric cancer stem cells ^{182,187} <i>NOTCH1</i> upregulated with intestinal-like phenotypes of gastric lesions ¹⁸³
<i>NOTCH2</i>	Correlated with intestinal and diffuse type gastric cancer ¹⁸³ Expression of <i>NOTCH2-NICD</i> promoted human gastric cancer cell proliferation and xenograft tumor growth ¹⁸⁸
<i>NOTCH3</i>	Expression increased in gastric tumors compared to normal, associated with intestinal/glandular differentiation of gastric carcinoma cells ¹⁸⁴
<i>NOTCH4</i>	Expression in gastric cancer cell line promoted growth ¹⁸⁹
<i>JAG1</i>	Expression increased in gastric tumors compared to normal ¹⁸⁴
<i>JAG2</i>	Expression increased in gastric tumors compared to normal, associated with intestinal/glandular differentiation of gastric carcinoma cells ¹⁸⁴
<i>DLL1</i>	Expression associated with diffuse and mixed type of gastric cancer ¹⁹⁰

Notch components are upregulated in gastric cancer. Definitive knowledge as to the role of specific Notch receptors and ligands in gastric tumorigenesis is still limited.

1.4: THESIS OVERVIEW

The Notch signaling pathway has been identified as a key regulator of gastric stem and progenitor cell homeostasis. Notch signaling promotes antral stem cell proliferation and directs cell fate choice, and constitutive Notch activation leads to gastric polyp formation^{10,11}. However, the receptors or ligands involved in Notch regulation of gastric stem cells have not been identified.

This thesis project aimed to describe the role of the Notch receptors in the regulation of gastric stem and epithelial cell dynamics. I have approached this using mouse models to map cells that are Notch signaling and determine where NOTCH1 and NOTCH2 are expressed. Functional analysis of the NOTCH1 and NOTCH2 receptors was investigated by pharmacologic inhibition of Notch receptors in mouse models. Mouse and human gastric organoids were used to uncover the role of the Notch receptors in epithelial cells.

In Chapter 2, I investigated the function of Notch receptors in antral epithelial differentiation and stem cell maintenance with inhibitory antibodies directed against NOTCH1 or NOTCH2. I found that combined inhibition of NOTCH1 and NOTCH2 mimics pan-Notch inhibition from γ -secretase inhibitor treatment. I showed reduced epithelial and stem cell proliferation and increased apoptosis. My studies showed that NOTCH1 and NOTCH2 are the primary Notch receptors regulating antral epithelial homeostasis. Inhibition of both NOTCH1 and NOTCH2 led to increased differentiation of

all gastric antral lineages. Notch inhibition also led to the unique finding that cells in the base and surface of the glands remodeled with Notch inhibition, increasing expression of secretory products from other regions of the gastrointestinal tract, including the corpus and intestine. Analysis of mouse and human organoids showed that Notch signaling through NOTCH1 and NOTCH2 is intrinsic to the epithelium and regulates growth of human and mouse gastric stem cells *in vitro*.

In Chapter 3, I investigated the role of Notch signaling in the maintenance of corpus epithelial cells using pharmacologic inhibition of the Notch signaling pathway and inhibitory antibodies against the NOTCH1 or NOTCH2 receptors. Similar to the antrum, inhibition of NOTCH1 and NOTCH2 mimics the reduction in proliferating epithelial cells seen with pan-Notch inhibition, indicating that these are the key receptors involved in corpus epithelial homeostasis. No changes to differentiated cell types were observed *in vivo*, but this may be a consequence of the longer turnover time of corpus epithelial cells coupled with the short timepoint of pharmacologic Notch inhibition required due to the lethality of global Notch inhibition. I observed a decrease in mouse and human corpus organoid growth with pan-Notch inhibition and NOTCH1 and/or NOTCH2 inhibition, indicating that Notch signaling is intrinsic to corpus epithelial cells.

In Chapter 4, I outline the protocol I established for mouse and human gastric organoid culture. I characterize growth conditions and a histological analysis of established organoids. I investigated the role of Notch signaling on mouse corpus and antral organoid growth and found that antral organoids are significantly more sensitive to Notch inhibition compared to corpus organoids.

In Chapter 5, I have put my work in the context of the field and demonstrate how these findings provide important insights into the regulation of gastric stem and epithelial cells. I propose future directions and experiments that will further characterize and understand the role of Notch signaling on maintenance of gastric stem cells and how those findings can be used in a therapeutic context.

REFERENCES

1. Koch U, Lehal R, Radtke F. Stem cells living with a Notch. *Development*. 2013;140(4):689-704. doi:10.1242/dev.080614.
2. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature*. 2005;435(June):959-963. doi:10.1038/nature03659.
3. VanDussen KL, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kolterud A, Grosse AS, Gumucio DL, Ernst S a, Tsai Y-H, Dempsey PJ, Samuelson LC. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. *Development*. 2012;139(3):488-497. doi:10.1242/dev.070763.
4. Fre S, Hannezo E, Sale S, Huyghe M, Lafkas D, Kissel H, Louvi A, Greve J, Louvard D, Artavanis-Tsakonas S. Notch lineages and activity in intestinal stem cells determined by a new set of knock-in mice. *PLoS One*. 2011;6(10):e25785. doi:10.1371/journal.pone.0025785.
5. Pellegrinet L, Rodilla V, Liu Z, Chen S, Koch U, Espinosa L, Kaestner KH, Kopan R, Lewis J, Radtke F. Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. *Gastroenterology*. 2011;140(4):1230-1240.e7. doi:10.1053/j.gastro.2011.01.005.
6. Riccio O, van Gijn ME, Bezdek AC, Pellegrinet L, van Es JH, Zimmer-Strobl U, Strobl LJ, Honjo T, Clevers H, Radtke F. Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression

- of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep.* 2008;9(4):377-383. doi:10.1038/embor.2008.7.
7. Stanger BZ, Datar R, Murtaugh LC, Melton D a. Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci U S A.* 2005;102(35):12443-12448. doi:10.1073/pnas.0505690102.
 8. Carulli AJ, Keeley TM, Demitrack ES, Chung J, Maillard I, Samuelson LC. Notch receptor regulation of intestinal stem cell homeostasis and crypt regeneration. *Dev Biol.* 2015;402(1):98-108. doi:10.1016/j.ydbio.2015.03.012.
 9. Tran IT, Sandy AR, Carulli AJ, Ebens C, Chung J, Shan GT, Radojcic V, Friedman A, Gridley T, Shelton A, Reddy P, Samuelson LC, Yan M, Siebel CW, Maillard I. Blockade of individual notch ligands and receptors controls graft-versus-host disease. *J Clin Invest.* 2013;123(4):1590-1604. doi:10.1172/JCI65477.
 10. Kim T-H, Shivdasani R a. Notch signaling in stomach epithelial stem cell homeostasis. *J Exp Med.* 2011;208(4):677-688. doi:10.1084/jem.20101737.
 11. Demitrack ES, Gifford GB, Keeley TM, Carulli AJ, VanDussen KL, Thomas D, Giordano TJ, Liu Z, Kopan R, Samuelson LC. Notch signaling regulates gastric antral LGR5 stem cell function. *EMBO J.* 2015;34(20):2522-2536. doi:10.15252/embj.201490583.
 12. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. II. Outward migration of pit cells. *Anat Rec.* 1993;236(2):280-296. doi:10.1002/ar.1092360203.
 13. Karam SM. Dynamics of epithelial cells in the corpus of the mouse stomach. IV. Bidirectional migration of parietal cells ending in their gradual degeneration and loss. *Anat Rec.* 1993;236(2):314-332. doi:10.1002/ar.1092360205.
 14. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. *Anat Rec.* 1993;236(2):259-279. doi:10.1002/ar.1092360202.
 15. Khurana S, Mills JC. *The Gastric Mucosa: Development and Differentiation.* Vol 96. Elsevier Inc.; 2010. doi:10.1016/B978-0-12-381280-3.00004-X.

16. Fukaya M, Isohata N, Ohta H, Aoyagi K, Ochiya T, Saeki N, Yanagihara K, Nakanishi Y, Taniguchi H, Sakamoto H, Shimoda T, Nimura Y, Yoshida T, Sasaki H. Hedgehog Signal Activation in Gastric Pit Cell and in Diffuse-Type Gastric Cancer. *Gastroenterology*. 2006;131(1):14-29. doi:10.1053/j.gastro.2006.05.008.
17. Verzi MP, Khan AH, Ito S, Shivdasani R a. Transcription Factor Foxq1 Controls Mucin Gene Expression and Granule Content in Mouse Stomach Surface Mucous Cells. *Gastroenterology*. 2008;135:591-600. doi:10.1053/j.gastro.2008.04.019.
18. Hirst BH, Forte JG. Redistribution and characterization of (H⁺ + K⁺)-ATPase membranes from resting and stimulated gastric parietal cells. *Biochem J*. 1985;231(3):641-649.
19. Stepan V, Ramamoorthy S, Nitsche H, Zavros Y, Merchant JL, Todisco A. Regulation and function of the sonic hedgehog signal transduction pathway in isolated gastric parietal cells. *J Biol Chem*. 2005;280(16):15700-15708. doi:10.1074/jbc.M413037200.
20. Shinohara M, Mao M, Keeley, Theresa M, El-zaatari M, Lee H-J, Eaton K a, Samuelson LC, Merchant JL, Goldenring JR, Todisco A. Bone Morphogenetic Protein Signaling Regulates Gastric Epithelial Cell Development and Proliferation in Mice. *Gastroenterology*. 2010;139(6):2050-2060. doi:10.1053/j.gastro.2010.
21. Ramsey VG, Doherty JM, Chen CC, Stappenbeck TS, Konieczny SF, Mills JC. The maturation of mucus-secreting gastric epithelial progenitors into digestive-enzyme secreting zymogenic cells requires Mist1. *Development*. 2007;134(1):211-222. doi:10.1242/dev.02700.
22. Karam SM. Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci*. 1999;4(1):D286-D298. doi:10.2741/Karam.
23. Tani S, Kobayashi S, Tanaka T. Secretion of intrinsic factor from cultured rat gastric chief cells. *Biol Pharm Bull*. 1994;17(10):1333-1336. <http://www.ncbi.nlm.nih.gov/pubmed/7874053>. Accessed October 12, 2015.
24. Nozaki K, Ogawa M, Williams JA, Lafleur BJ, Ng V, Drapkin RI, Mills JC, Konieczny SF, Nomura S, Goldenring JR. A molecular signature of gastric metaplasia arising in response to acute parietal cell loss. *Gastroenterology*. 2008;134(2):511-522. doi:10.1053/j.gastro.2007.11.058.

25. Meuwissen SG, Bosma A, van Donk E, Waalewijn R, Pals G, Pronk JC, Eriksson AW, Mullink H, Meijer CJ. Immunohistochemical localization of pepsinogen A and C containing cells in Barrett's oesophagus. *Virchows Arch A Pathol Anat Histopathol*. 1988;413(1):11-16.
26. Facer P, Bishop AE, Lloyd R V, Wilson BS, Hennessy RJ, Polak JM. Chromogranin: a newly recognized marker for endocrine cells of the human gastrointestinal tract. *Gastroenterology*. 1985;89(6):1366-1373.
27. Chu S, Schubert ML. Gastric secretion. *Curr Opin Gastroenterol*. 2012;28(6):587-593. doi:10.1097/MOG.0b013e328358e5cc.
28. Li HJ, Johnston B, Aiello D, Caffrey DR, Giel-Moloney M, Rindi G, Leiter AB. Distinct Cellular Origins for Serotonin-Expressing and Enterochromaffin-like Cells in the Gastric Corpus. *Gastroenterology*. 2014;146(3):754-764.e3. doi:10.1053/j.gastro.2013.11.048.
29. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R, Clevers H. Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. *Cell Stem Cell*. 2010;6(1):25-36. doi:10.1016/j.stem.2009.11.013.
30. Lee ER, Leblond CP. Dynamic histology of the antral epithelium in the mouse stomach: IV. Ultrastructure and renewal of gland cells. *Am J Anat*. 1985;172(3):241-259.
31. Schonhoff SE, Giel-Moloney M, Leiter AB. Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol*. 2004;270(2):443-454. doi:10.1016/j.ydbio.2004.03.013.
32. Horst D, Gu X, Bhasin M, Yang Q, Verzi M, Lin D, Joseph M, Zhang X, Chen W, Li Y-P, Shivdasani R a, Libermann T a. Requirement of the epithelium-specific Ets transcription factor Spdef for mucous gland cell function in the gastric antrum. *J Biol Chem*. 2010;285(45):35047-35055. doi:10.1074/jbc.M110.164541.
33. Choi E, Roland JT, Barlow BJ, O'Neal R, Rich AE, Nam KT, Shi C, Goldenring JR. Cell lineage distribution atlas of the human stomach reveals heterogeneous gland populations in the gastric antrum. *Gut*. 2014:1-10. doi:10.1136/gutjnl-2013-305964.

34. Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. *Science*. 2010;327(5965):542-545. doi:10.1126/science.1180794.
35. Stange DE, Koo BK, Huch M, Sibbel G, Basak O, Lyubimova A, Kujala P, Bartfeld S, Koster J, Geahlen JH, Peters PJ, Van Es JH, Van De Wetering M, Mills JC, Clevers H. Differentiated Troy⁺ chief cells act as reserve stem cells to generate all lineages of the stomach epithelium. *Cell*. 2013;155(2):357-368. doi:10.1016/j.cell.2013.09.008.
36. Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Mary K, Higginbotham JN, Juchheim A, Prasad N, Shawn E, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, Coffey RJ. The pan-ErbB negative regulator, Lrig1, is an intestinal stem cell marker that functions as a tumor suppressor. *Cell*. 2012;149(1):146-158. doi:10.1016/j.cell.2012.02.042.The.
37. Qiao XT, Ziel JW, McKimpson W, Madison BB, Todisco A, Merchant JL, Samuelson LC, Gumucio DL. Prospective identification of a multilineage progenitor in murine stomach epithelium. *Gastroenterology*. 2007;133(6):1989-1998. doi:10.1053/j.gastro.2007.09.031.
38. Leblond CP, Stevens CE, Bogoroch R. Histological Localization of Newly-formed Desoxyribonucleic Acid. *Science*. 1948;108(2811):531-533. doi:10.1126/science.108.2811.531.
39. Corpron RE. The ultrastructure of the gastric mucosa in normal and hypophysectomized rats. *Am J Anat*. 1966;118(1):53-90. doi:10.1002/aja.1001180105.
40. Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD, Clevers H. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell*. 2010;143(1):134-144. doi:10.1016/j.cell.2010.09.016.
41. Nomura S, Esumi H, Job C, Tan SS. Lineage and clonal development of gastric glands. *Dev Biol*. 1998;204(1):124-135. doi:10.1006/dbio.1998.9055.
42. Leushacke M, Ng A, Galle J, Loeffler M, Barker N. Lgr5(+) gastric stem cells divide symmetrically to effect epithelial homeostasis in the pylorus. *Cell Rep*.

- 2013;5(2):349-356. doi:10.1016/j.celrep.2013.09.025.
43. Kretzschmar K, Watt FM. Lineage tracing. *Cell*. 2012;148(1-2):33-45. doi:10.1016/j.cell.2012.01.002.
 44. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. 2009;459(7244):262-265. doi:10.1038/nature07935.
 45. Huch M, Dorrell C, Boj SF, van Es JH, Li VSW, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, Haft A, Vries RG, Grompe M, Clevers H. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature*. 2013;494(7436):247-250. doi:10.1038/nature11826.
 46. Huch M, Bonfanti P, Boj SF, Sato T, Loomans CJM, van de Wetering M, Sojoodi M, Li VSW, Schuijers J, Gracanin A, Ringnald F, Begthel H, Hamer K, Mulder J, van Es JH, de Koning E, Vries RGJ, Heimberg H, Clevers H. Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *EMBO J*. 2013;32(20):2708-2721. doi:10.1038/emboj.2013.204.
 47. Sato T, Stange DE, Ferrante M, Vries RGJ, Van Es JH, Van Den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD, Clevers H. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. 2011;141(5):1762-1772. doi:10.1053/j.gastro.2011.07.050.
 48. VanDussen KL, Marinshaw JM, Shaikh N, Miyoshi H, Moon C, Tarr PI, Ciorba MA, Stappenbeck TS. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut*. 2015;64(6):911-920. doi:10.1136/gutjnl-2013-306651.
 49. Nam KT, O'Neal RL, Coffey RJ, Finke PE, Barker N, Goldenring JR. Spasmolytic polypeptide-expressing metaplasia (SPEM) in the gastric oxyntic mucosa does not arise from Lgr5-expressing cells. *Gut*. 2012;61(12):1678-1685. doi:10.1136/gutjnl-2011-301193.
 50. Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N, Hochedlinger K. Sox2 + adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell*. 2011;9(4):317-329. doi:10.1016/j.stem.2011.09.001.

51. Hayakawa Y, Jin G, Wang H, Chen X, Westphalen CB, Asfaha S, Renz BW, Ariyama H, Dubeykovskaya ZA, Takemoto Y, Lee Y, Muley A, Tailor Y, Chen D, Muthupalani S, Fox JG, Shulkes A, Worthley DL, Takaishi S, Wang TC. CCK2R identifies and regulates gastric antral stem cell states and carcinogenesis. *Gut*. 2015;64(4):544-553. doi:10.1136/gutjnl-2014-307190.
52. Wong VWY, Stange DE, Page ME, Buczacki S, Wabik A, Itami S, van de Wetering M, Poulsom R, Wright N a., Trotter MWB, Watt FM, Winton DJ, Clevers H, Jensen KB. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nat Cell Biol*. 2012;14(4):401-408. doi:10.1038/ncb2464.
53. Quante M, Marrache F, Goldenring JR, Wang TC. TFF2 mRNA transcript expression marks a gland progenitor cell of the gastric oxyntic mucosa. *Gastroenterology*. 2010;139(6):2018-2027. doi:10.1016/j.biotechadv.2011.08.021.Secreted.
54. Nam KT, Lee H, Sousa JF, Weis VG, O'Neal RL, Finke PE, Romerogallo J, Shi G, Mills JC, Peek RM, Konieczny SF, Goldenring JR. Mature chief cells are cryptic progenitors for metaplasia in the stomach. *Gastroenterology*. 2010;139(6):2028-2037.e9. doi:10.1053/j.gastro.2010.09.005.
55. Ramalho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development*. 2000;127(12):2763-2772.
56. Ohta H, Aoyagi K, Fukaya M, Danjoh I, Ohta A, Isohata N, Saeki N, Taniguchi H, Sakamoto H, Shimoda T, Tani T, Yoshida T, Sasaki H. Cross talk between hedgehog and epithelial–mesenchymal transition pathways in gastric pit cells and in diffuse-type gastric cancers. *Br J Cancer*. 2008;100(2):389-398. doi:10.1038/sj.bjc.6604846.
57. Waghray M, Zavros Y, Saqui-Salces M, El-Zaatari M, Alamelumangapuram CB, Todisco A, Eaton KA, Merchant JL. Interleukin-1beta promotes gastric atrophy through suppression of Sonic Hedgehog. *Gastroenterology*. 2010;138(2):562-572, 572.e1-e2. doi:10.1053/j.gastro.2009.10.043.
58. Xiao C, Ogle SA, Schumacher MA, Orr–Asman MA, Miller ML, Lertkowitz N, Varro A, Hollande F, Zavros Y. Loss of Parietal Cell Expression of Sonic Hedgehog Induces Hypergastrinemia and Hyperproliferation of Surface Mucous Cells.

Gastroenterology. 2010;138(2):550-561.e8. doi:10.1053/j.gastro.2009.11.002.

59. Radulescu S, Ridgway R a, Cordero J, Athineos D, Salgueiro P, Poulsom R, Neumann J, Jung a, Patel S, Woodgett J, Barker N, Pritchard DM, Oien K, Sansom OJ. Acute WNT signalling activation perturbs differentiation within the adult stomach and rapidly leads to tumour formation. *Oncogene*. 2012;32(16):2048-2057. doi:10.1038/onc.2012.224.
60. van den Brink GR, Hardwick JCH, Tytgat GNJ, Brink MA, Ten Kate FJ, Van Deventer SJH, Peppelenbosch MP, Van Deventer SJH, Tytgat GNJ, Brink MA, Ten Kate FJ. Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology*. 2001;121(2):317-328. doi:10.1053/gast.2001.26261.
61. Maloum F, Allaire JM, Gagne-Sansfacon J, Roy E, Belleville K, Sarret P, Morisset J, Carrier JC, Mishina Y, Kaestner KH, Perreault N. Epithelial BMP signaling is required for proper specification of epithelial cell lineages and gastric endocrine cells. *Am J Physiol Gastrointest Liver Physiol*. 2011;300(6):G1065-G1079. doi:10.1152/ajpgi.00176.2010.
62. Coffey RJ, Romano M, Goldenring J. Roles for transforming growth factor-alpha in the stomach. *J Clin Gastroenterol*. 1995;21 Suppl 1:S36-S39.
63. Sferra TJ, Pawel BR, Qualman SJ, Li BU. Ménétrier disease of childhood: role of cytomegalovirus and transforming growth factor alpha. *J Pediatr*. 1996;128(2):213-219.
64. Ohning G V, Wong HC, Lloyd KC, Walsh JH. Gastrin mediates the gastric mucosal proliferative response to feeding. *Am J Physiol*. 1996;271(3 Pt 1):G470-G476.
65. Kazumori H, Ishihara S, Kawashima K, Fukuda R, Chiba T, Kinoshita Y. Analysis of gastrin receptor gene expression in proliferating cells in the neck zone of gastric fundic glands using laser capture microdissection. *FEBS Lett*. 2001;489(2-3):208-214.
66. Ohata S, Aoki R, Kinoshita S, Yamaguchi M, Tsuruoka-Kinoshita S, Tanaka H, Wada H, Watabe S, Tsuboi T, Masai I, Okamoto H. Dual roles of Notch in regulation of apically restricted mitosis and apicobasal polarity of neuroepithelial cells. *Neuron*. 2011;69(2):215-230. doi:10.1016/j.neuron.2010.12.026.

67. Tanigaki K, Honjo T. Two opposing roles of RBP-J in Notch signaling. *Curr Top Dev Biol.* 2010;92:231-252. doi:10.1016/S0070-2153(10)92007-3.
68. Buono KD, Robinson GW, Martin C, Shi S, Stanley P, Tanigaki K, Honjo T, Hennighausen L. The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy. *Dev Biol.* 2006;293(2):565-580. doi:10.1016/j.ydbio.2006.02.043.
69. Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze D V, Weinmaster G, Gridley T. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* 1998;12(7):1046-1057.
70. Casey LM, Lan Y, Cho E-S, Maltby KM, Gridley T, Jiang R. Jag2-Notch1 signaling regulates oral epithelial differentiation and palate development. *Dev Dyn.* 2006;235(7):1830-1844. doi:10.1002/dvdy.20821.
71. Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont ME, Rand EB, Piccoli DA, Hood L, Spinner NB. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet.* 1997;16(3):243-251. doi:10.1038/ng0797-243.
72. Mitsiadis TA, Regaudiat L, Gridley T. Role of the Notch signalling pathway in tooth morphogenesis. *Arch Oral Biol.* 2005;50(2):137-140. doi:10.1016/j.archoralbio.2004.10.006.
73. Loomes KM, Stevens SA, O'Brien ML, Gonzalez DM, Ryan MJ, Segalov M, Dormans NJ, Mimoto MS, Gibson JD, Sewell W, Schaffer AA, Nah H-D, Rappaport EF, Pratt SC, Dunwoodie SL, Kusumi K. Dll3 and Notch1 genetic interactions model axial segmental and craniofacial malformations of human birth defects. *Dev Dyn.* 2007;236(10):2943-2951. doi:10.1002/dvdy.21296.
74. Yamamoto N, Chang W, Kelley MW. Rbpj regulates development of prosensory cells in the mammalian inner ear. *Dev Biol.* 2011;353(2):367-379. doi:10.1016/j.ydbio.2011.03.016.
75. Kiernan AE, Xu J, Gridley T. The Notch Ligand JAG1 Is Required for Sensory Progenitor Development in the Mammalian Inner Ear. *PLoS Genet.* 2006;2(1):e4. doi:10.1371/journal.pgen.0020004.
76. Cotanche DA, Kaiser CL. Hair cell fate decisions in cochlear development and

- regeneration. *Hear Res.* 2010;266(1-2):18-25. doi:10.1016/j.heares.2010.04.012.
77. Ohashi S, Natsuizaka M, Yashiro-Ohtani Y, Kalman RA, Nakagawa M, Wu L, Klein-Szanto AJ, Herlyn M, Diehl JA, Katz JP, Pear WS, Seykora JT, Nakagawa H. NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network. *Gastroenterology.* 2010;139(6):2113-2123. doi:10.1053/j.gastro.2010.08.040.
 78. Rowan S, Conley KW, Le TT, Donner AL, Maas RL, Brown NL. Notch signaling regulates growth and differentiation in the mammalian lens. *Dev Biol.* 2008;321(1):111-122. doi:10.1016/j.ydbio.2008.06.002.
 79. Jia J, Lin M, Zhang L, York JP, Zhang P. The Notch signaling pathway controls the size of the ocular lens by directly suppressing p57Kip2 expression. *Mol Cell Biol.* 2007;27(20):7236-7247. doi:10.1128/MCB.00780-07.
 80. Le TT, Conley KW, Brown NL. Jagged 1 is necessary for normal mouse lens formation. *Dev Biol.* 2009;328(1):118-126. doi:10.1016/j.ydbio.2009.01.015.
 81. MacGrogan D, Nus M, de la Pompa JL. *Notch Signaling.* Vol 92. Elsevier; 2010. doi:10.1016/S0070-2153(10)92011-5.
 82. Bigas A, Robert-Moreno A, Espinosa L. The Notch pathway in the developing hematopoietic system. *Int J Dev Biol.* 2010;54(6-7):1175-1188. doi:10.1387/ijdb.093049ab.
 83. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S. Notch signals control the fate of immature progenitor cells in the intestine. *Nature.* 2005;435(7044):964-968. doi:10.1038/nature03589.
 84. Cheng H-T, Kim M, Valerius MT, Surendran K, Schuster-Gossler K, Gossler A, McMahon AP, Kopan R. Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development.* 2007;134(4):801-811. doi:10.1242/dev.02773.
 85. Pan Y, Liu Z, Shen J, Kopan R. Notch1 and 2 cooperate in limb ectoderm to receive an early Jagged2 signal regulating interdigital apoptosis. *Dev Biol.* 2005;286(2):472-482. doi:10.1016/j.ydbio.2005.08.037.

86. Francis JC, Radtke F, Logan MPO. Notch1 signals through Jagged2 to regulate apoptosis in the apical ectodermal ridge of the developing limb bud. *Dev Dyn*. 2005;234(4):1006-1015. doi:10.1002/dvdy.20590.
87. McGlinn E, van Bueren KL, Fiorenza S, Mo R, Poh AM, Forrest A, Soares MB, Bonaldo M de F, Grimmond S, Hui C-C, Wainwright B, Wicking C. Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. *Mech Dev*. 2005;122(11):1218-1233. doi:10.1016/j.mod.2005.06.012.
88. Vasiliauskas D, Laufer E, Stern CD. A role for hairy1 in regulating chick limb bud growth. *Dev Biol*. 2003;262(1):94-106.
89. Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, Radtke F, Schmid RM, Siveke JT. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. *Hepatology*. 2008;48(2):607-616. doi:10.1002/hep.22381.
90. Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, Stanger BZ. Notch signaling controls liver development by regulating biliary differentiation. *Development*. 2009;136(10):1727-1739. doi:10.1242/dev.029140.
91. Lozier J, McCright B, Gridley T. Notch Signaling Regulates Bile Duct Morphogenesis in Mice. Lopez-Schier H, ed. *PLoS One*. 2008;3(3):e1851. doi:10.1371/journal.pone.0001851.
92. Ghabrial AS, Krasnow MA. Social interactions among epithelial cells during tracheal branching morphogenesis. *Nature*. 2006;441(7094):746-749. doi:10.1038/nature04829.
93. Tsvitse S. Notch and Wnt signaling, physiological stimuli and postnatal myogenesis. *Int J Biol Sci*. 2010;6(3):268-281.
94. Jain R, Engleka KA, Rentschler SL, Manderfield LJ, Li L, Yuan L, Epstein JA. Cardiac neural crest orchestrates remodeling and functional maturation of mouse semilunar valves. *J Clin Invest*. 2011;121(1):422-430. doi:10.1172/JCI44244.
95. Mirsky R, Woodhoo A, Parkinson DB, Arthur-Farraj P, Bhaskaran A, Jessen KR. Novel signals controlling embryonic Schwann cell development, myelination and dedifferentiation. *J Peripher Nerv Syst*. 2008;13(2):122-135. doi:10.1111/j.1529-8027.2008.00168.x.

96. Schouwey K, Beermann F. The Notch pathway: hair graying and pigment cell homeostasis. *Histol Histopathol.* 2008;23(5):609-619.
97. Kim W, Shin Y-K, Kim B-J, Egan JM. Notch signaling in pancreatic endocrine cell and diabetes. *Biochem Biophys Res Commun.* 2010;392(3):247-251. doi:10.1016/j.bbrc.2009.12.115.
98. Monahan P, Rybak S, Raetzman LT. The notch target gene HES1 regulates cell cycle inhibitor expression in the developing pituitary. *Endocrinology.* 2009;150(9):4386-4394. doi:10.1210/en.2009-0206.
99. Raetzman LT, Cai JX, Camper SA. Hes1 is required for pituitary growth and melanotrope specification. *Dev Biol.* 2007;304(2):455-466. doi:10.1016/j.ydbio.2006.11.010.
100. Davis SW, Castinetti F, Carvalho LR, Ellsworth BS, Potok MA, Lyons RH, Brinkmeier ML, Raetzman LT, Carninci P, Mortensen AH, Hayashizaki Y, Arnhold IJP, Mendonça BB, Brue T, Camper SA. Molecular mechanisms of pituitary organogenesis: In search of novel regulatory genes. *Mol Cell Endocrinol.* 2010;323(1):4-19. doi:10.1016/j.mce.2009.12.012.
101. Gasperowicz M, Otto F. The Notch Signalling Pathway in the Development of the Mouse Placenta. *Placenta.* 2008;29(8):651-659. doi:10.1016/j.placenta.2008.06.004.
102. Wang X-D, Leow CC, Zha J, Tang Z, Modrusan Z, Radtke F, Aguet M, de Sauvage FJ, Gao W-Q. Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation. *Dev Biol.* 2006;290(1):66-80. doi:10.1016/j.ydbio.2005.11.009.
103. Wang X-D, Shou J, Wong P, French DM, Gao W-Q. Notch1-expressing cells are indispensable for prostatic branching morphogenesis during development and re-growth following castration and androgen replacement. *J Biol Chem.* 2004;279(23):24733-24744. doi:10.1074/jbc.M401602200.
104. Orr B, Grace OC, Vanpoucke G, Ashley GR, Thomson AA. A Role for Notch Signaling in Stromal Survival and Differentiation during Prostate Development. *Endocrinology.* 2009;150(1):463-472. doi:10.1210/en.2008-0383.

105. Tang H, Brennan J, Karl J, Hamada Y, Raetzman L, Capel B. Notch signaling maintains Leydig progenitor cells in the mouse testis. *Development*. 2008;135(22):3745-3753. doi:10.1242/dev.024786.
106. Hayashi T, Kageyama Y, Ishizaka K, Xia G, Kihara K, Oshima H. Requirement of Notch 1 and its ligand jagged 2 expressions for spermatogenesis in rat and human testes. *J Androl*. 22(6):999-1011.
107. Nadarajan S, Govindan JA, McGovern M, Hubbard EJA, Greenstein D. MSP and GLP-1/Notch signaling coordinately regulate actomyosin-dependent cytoplasmic streaming and oocyte growth in *C. elegans*. *Development*. 2009;136(13):2223-2234. doi:10.1242/dev.034603.
108. Barsoum IB, Yao HH-C. Fetal Leydig Cells: Progenitor Cell Maintenance and Differentiation. *J Androl*. 2009;31(1):11-15. doi:10.2164/jandrol.109.008318.
109. Massi D, Panelos J. Notch signaling and the developing skin epidermis. *Adv Exp Med Biol*. 2012;727:131-141. doi:10.1007/978-1-4614-0899-4_10.
110. Aubin-Houzelstein G. Notch signaling and the developing hair follicle. *Adv Exp Med Biol*. 2012;727:142-160. doi:10.1007/978-1-4614-0899-4_11.
111. Dunwoodie SL. The role of Notch in patterning the human vertebral column. *Curr Opin Genet Dev*. 2009;19(4):329-337. doi:10.1016/j.gde.2009.06.005.
112. Kageyama R, Niwa Y, Shimojo H, Kobayashi T, Ohtsuka T. Ultradian oscillations in Notch signaling regulate dynamic biological events. *Curr Top Dev Biol*. 2010;92:311-331. doi:10.1016/S0070-2153(10)92010-3.
113. Yuan JS, Kousis PC, Suliman S, Visan I, Guidos CJ. Functions of notch signaling in the immune system: consensus and controversies. *Annu Rev Immunol*. 2010;28:343-365. doi:10.1146/annurev.immunol.021908.132719.
114. Matsuda Y, Wakamatsu Y, Kohyama J, Okano H, Fukuda K, Yasugi S. Notch signaling functions as a binary switch for the determination of glandular and luminal fates of endodermal epithelium during chicken stomach development. *Development*. 2005;132(12):2783-2793. doi:10.1242/dev.01853.
115. Carre A, Rachdi L, Tron E, Richard B, Castanet M, Schlumberger M, Bidart J-M,

- Szinnai G, Polak M. Hes1 is required for appropriate morphogenesis and differentiation during mouse thyroid gland development. *PLoS One*. 2011;6(2):e16752. doi:10.1371/journal.pone.0016752.
116. Gridley T. Notch signaling in the vasculature. *Curr Top Dev Biol*. 2010;92:277-309. doi:10.1016/S0070-2153(10)92009-7.
 117. Liu J, Sato C, Cerletti M, Wagers A. Notch signaling in the regulation of stem cell self-renewal and differentiation. *Curr Top Dev Biol*. 2010;92:367-409. doi:10.1016/S0070-2153(10)92012-7.
 118. Andersson ER, Sandberg R, Lendahl U. Notch signaling: simplicity in design, versatility in function. *Development*. 2011;138(17):3593-3612. doi:10.1242/dev.063610.
 119. D'Souza B, Meloty-Kapella L, Weinmaster G. Canonical and non-canonical Notch ligands. *Curr Top Dev Biol*. 2010;92:73-129. doi:10.1016/S0070-2153(10)92003-6.
 120. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006;7(9):678-689. doi:10.1038/nrm2009.
 121. Kopan R, Ilagan MXG. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*. 2009;137(2):216-233. doi:10.1016/j.cell.2009.03.045.
 122. Hori K, Sen a., Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci*. 2013;126:2135-2140. doi:10.1242/jcs.127308.
 123. Logeat F, Bessia C, Brou C, LeBail O, Jarriault S, Seidah NG, Israël A. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A*. 1998;95(14):8108-8112.
 124. Kopan R, Schroeter EH, Weintraub H, Nye JS. Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc Natl Acad Sci U S A*. 1996;93(4):1683-1688.
 125. Lieber T, Kidd S, Alcamo E, Corbin V, Young MW. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev*. 1993;7(10):1949-1965.

<http://www.ncbi.nlm.nih.gov/pubmed/8406001>. Accessed October 13, 2015.

126. Rebay I, Fehon RG, Artavanis-Tsakonas S. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. *Cell*. 1993;74(2):319-329.
127. Greenwald I, Seydoux G. Analysis of gain-of-function mutations of the lin-12 gene of *Caenorhabditis elegans*. *Nature*. 1990;346(6280):197-199. doi:10.1038/346197a0.
128. Parks AL, Klueg KM, Stout JR, Muskavitch MA. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development*. 2000;127(7):1373-1385.
129. Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, Cumano A, Roux P, Black RA, Israël A. A Novel Proteolytic Cleavage Involved in Notch Signaling. *Mol Cell*. 2000;5(2):207-216. doi:10.1016/S1097-2765(00)80417-7.
130. Canault M, Certel K, Schatzberg D, Wagner DD, Hynes RO. The lack of ADAM17 activity during embryonic development causes hemorrhage and impairs vessel formation. *PLoS One*. 2010;5(10):e13433. doi:10.1371/journal.pone.0013433.
131. Tian L, Wu X, Chi C, Han M, Xu T, Zhuang Y. ADAM10 is essential for proteolytic activation of Notch during thymocyte development. *Int Immunol*. 2008;20(9):1181-1187. doi:10.1093/intimm/dxn076.
132. Tousseyn T, Thathiah A, Jorissen E, Raemaekers T, Konietzko U, Reiss K, Maes E, Snellinx A, Serneels L, Nyabi O, Annaert W, Saftig P, Hartmann D, De Strooper B. ADAM10, the rate-limiting protease of regulated intramembrane proteolysis of Notch and other proteins, is processed by ADAMS-9, ADAMS-15, and the gamma-secretase. *J Biol Chem*. 2009;284(17):11738-11747. doi:10.1074/jbc.M805894200.
133. van Tetering G, van Diest P, Verlaan I, van der Wall E, Kopan R, Vooijs M. Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. *J Biol Chem*. 2009;284(45):31018-31027. doi:10.1074/jbc.M109.006775.
134. Rooke J, Pan D, Xu T, Rubin GM. KUZ, a conserved metalloprotease-disintegrin protein with two roles in Drosophila neurogenesis. *Science*. 1996;273(5279):1227-1231.

135. Hansson EM, Lanner F, Das D, Mutvei A, Marklund U, Ericson J, Farnebo F, Stumm G, Stenmark H, Andersson ER, Lendahl U. Control of Notch-ligand endocytosis by ligand-receptor interaction. *J Cell Sci.* 2010;123(Pt 17):2931-2942. doi:10.1242/jcs.073239.
136. De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ, Goate A, Kopan R. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature.* 1999;398(6727):518-522. doi:10.1038/19083.
137. Inglés-Esteve J, Espinosa L, Milner LA, Caelles C, Bigas A. Phosphorylation of Ser2078 modulates the Notch2 function in 32D cell differentiation. *J Biol Chem.* 2001;276(48):44873-44880. doi:10.1074/jbc.M104703200.
138. Espinosa L, Inglés-Esteve J, Aguilera C, Bigas A. Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *J Biol Chem.* 2003;278(34):32227-32235. doi:10.1074/jbc.M304001200.
139. Fryer CJ, White JB, Jones KA. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell.* 2004;16(4):509-520. doi:10.1016/j.molcel.2004.10.014.
140. Gupta-Rossi N, Le Bail O, Gonen H, Brou C, Logeat F, Six E, Ciechanover A, Israël A. Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. *J Biol Chem.* 2001;276(37):34371-34378. doi:10.1074/jbc.M101343200.
141. Tsunematsu R, Nakayama K, Oike Y, Nishiyama M, Ishida N, Hatakeyama S, Bessho Y, Kageyama R, Suda T, Nakayama KI. Mouse Fbw7/Sel-10/Cdc4 is required for notch degradation during vascular development. *J Biol Chem.* 2004;279(10):9417-9423. doi:10.1074/jbc.M312337200.
142. Fior R, Henrique D. A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol.* 2005;281(2):318-333. doi:10.1016/j.ydbio.2005.03.017.
143. Cooper MT, Tyler DM, Furriols M, Chalkiadaki A, Delidakis C, Bray S. Spatially restricted factors cooperate with notch in the regulation of Enhancer of split

- genes. *Dev Biol.* 2000;221(2):390-403. doi:10.1006/dbio.2000.9691.
144. Krejčí A, Bernard F, Housden BE, Collins S, Bray SJ. Direct response to Notch activation: signaling crosstalk and incoherent logic. *Sci Signal.* 2009;2(55):ra1. doi:10.1126/scisignal.2000140.
 145. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, Barnes KC, O'Neil J, Neuberg D, Weng AP, Aster JC, Sigaux F, Soulier J, Look AT, Young RA, Califano A, Ferrando AA. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci U S A.* 2006;103(48):18261-18266. doi:10.1073/pnas.0606108103.
 146. Jeffries S, Robbins DJ, Capobianco AJ. Characterization of a High-Molecular-Weight Notch Complex in the Nucleus of Notchic-Transformed RKE Cells and in a Human T-Cell Leukemia Cell Line. *Mol Cell Biol.* 2002;22(11):3927-3941. doi:10.1128/MCB.22.11.3927-3941.2002.
 147. Ronchini C, Capobianco AJ. Induction of Cyclin D1 Transcription and CDK2 Activity by Notchic: Implication for Cell Cycle Disruption in Transformation by Notchic. *Mol Cell Biol.* 2001;21(17):5925-5934. doi:10.1128/MCB.21.17.5925-5934.2001.
 148. Perdigoto CN, Bardin AJ. Sending the right signal: Notch and stem cells. *Biochim Biophys Acta.* 2013;1830(2):2307-2322. doi:10.1016/j.bbagen.2012.08.009.
 149. Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *EMBO J.* 2001;20(13):3427-3436. doi:10.1093/emboj/20.13.3427.
 150. Blanpain C, Lowry WE, Pasolli HA, Fuchs E. Canonical notch signaling functions as a commitment switch in the epidermal lineage. *Genes Dev.* 2006;20(21):3022-3035. doi:10.1101/gad.1477606.
 151. Demehri S, Turkoz A, Kopan R. Epidermal Notch1 Loss Promotes Skin Tumorigenesis by Impacting the Stromal Microenvironment. *Cancer Cell.* 2009;16(1):55-66. doi:10.1016/j.ccr.2009.05.016.
 152. MAURO A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol.* 1961;9:493-495.

153. Mourikis P, Sambasivan R, Castel D, Rocheteau P, Bizzarro V, Tajbakhsh S. A critical requirement for notch signaling in maintenance of the quiescent skeletal muscle stem cell state. *Stem Cells*. 2012;30(2):243-252. doi:10.1002/stem.775.
154. Bjornson CRR, Cheung TH, Liu L, Tripathi P V, Steeper KM, Rando TA. Notch signaling is necessary to maintain quiescence in adult muscle stem cells. *Stem Cells*. 2012;30(2):232-242. doi:10.1002/stem.773.
155. Conboy IM, Rando TA. The Regulation of Notch Signaling Controls Satellite Cell Activation and Cell Fate Determination in Postnatal Myogenesis. *Dev Cell*. 2002;3(3):397-409. doi:10.1016/S1534-5807(02)00254-X.
156. Sun D, Li H, Zolkiewska A. The role of Delta-like 1 shedding in muscle cell self-renewal and differentiation. *J Cell Sci*. 2008;121(22):3815-3823. doi:10.1242/jcs.035493.
157. Kopan R, Nye JS, Weintraub H. The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development*. 1994;120(9):2385-2396.
158. Kitzmann M, Bonnieu A, Duret C, Vernus B, Barro M, Laoudj-Chenivesse D, Verdi JM, Carnac G. Inhibition of Notch signaling induces myotube hypertrophy by recruiting a subpopulation of reserve cells. *J Cell Physiol*. 2006;208(3):538-548. doi:10.1002/jcp.20688.
159. Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E, Yamaguchi T, Masuda S, Shimizu K, Takahashi T, Ogawa S, Hamada Y, Hirai H. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity*. 2003;18(5):699-711.
160. Robert-Moreno A, Guiu J, Ruiz-Herguido C, López ME, Inglés-Esteve J, Riera L, Tipping A, Enver T, Dzierzak E, Gridley T, Espinosa L, Bigas A. Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. *EMBO J*. 2008;27(13):1886-1895. doi:10.1038/emboj.2008.113.
161. Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, Pear WS, Bernstein ID. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med*. 2000;6(11):1278-

1281. doi:10.1038/81390.
162. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity*. 1999;10(5):547-558.
163. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC, Pear WS. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity*. 1999;11(3):299-308.
164. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *Am J Physiol Lung Cell Mol Physiol*. 2004;286(4):L643-L649. doi:10.1152/ajplung.00155.2003.
165. Rock JR, Gao X, Xue Y, Randell SH, Kong Y-Y, Hogan BLM. Notch-dependent differentiation of adult airway basal stem cells. *Cell Stem Cell*. 2011;8(6):639-648. doi:10.1016/j.stem.2011.04.003.
166. Penton AL, Leonard LD, Spinner NB. Notch signaling in human development and disease. *Semin Cell Dev Biol*. 2012;23(4):450-457. doi:10.1016/j.semcdb.2012.01.010.
167. Fiúza U-M, Arias AM. Cell and molecular biology of Notch. *J Endocrinol*. 2007;194(3):459-474. doi:10.1677/JOE-07-0242.
168. Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadiant R, Jacobs RT, Zacco A, Greenberg B, Ciaccio PJ. Modulation of Notch processing by γ -secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci*. 2004;82(1):341-358. doi:10.1093/toxsci/kfh254.
169. Wong GT, Manfra D, Poulet FM, Zhang Q, Josien H, Bara T, Engstrom L, Pinzon-Ortiz M, Fine JS, Lee HJJ, Zhang L, Higgins G a., Parker EM. Chronic Treatment with the γ -Secretase Inhibitor LY-411,575 Inhibits γ -Amyloid Peptide Production and Alters Lymphopoiesis and Intestinal Cell Differentiation. *J Biol Chem*. 2004;279(13):12876-12882. doi:10.1074/jbc.M311652200.
170. VanDussen KL, Samuelson LC. Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate. *Dev Biol*.

2010;346(2):215-223. doi:10.1016/j.ydbio.2010.07.026.

171. Shroyer NF, Helmrath M a, Wang VY-C, Antalffy B, Henning SJ, Zoghbi HY. Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. *Gastroenterology*. 2007;132(7):2478-2488. doi:10.1053/j.gastro.2007.03.047.
172. van der Flier LG, Haegebarth A, Stange DE, van de Wetering M, Clevers H. OLFM4 Is a Robust Marker for Stem Cells in Human Intestine and Marks a Subset of Colorectal Cancer Cells. *Gastroenterology*. 2009;137(1):15-17. doi:10.1053/j.gastro.2009.05.035.
173. Sander GR, Powell BC. Expression of notch receptors and ligands in the adult gut. *J Histochem Cytochem*. 2004;52(4):509-516. doi:10.1177/002215540405200409.
174. Shroder N, Gossler A. Expression of Notch pathway components in fetal and adult mouse small intestine. *Gene Expr Patterns*. 2002;2:247-250. doi:10.1016/S1.
175. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, Finkle D, Venook R, Wu X, Ridgway J, Schahin-Reed D, Dow GJ, Shelton A, Stawicki S, Watts RJ, Zhang J, Choy R, Howard P, Kadyk L, Yan M, Zha J, Callahan C a, Hymowitz SG, Siebel CW. Therapeutic antibody targeting of individual Notch receptors. *Nature*. 2010;464(7291):1052-1057. doi:10.1038/nature08878.
176. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*. 2011;469(7330):415-418. doi:10.1038/nature09637.
177. van Es JH, Sato T, van de Wetering M, Lyubimova A, Nee ANY, Gregorieff A, Sasaki N, Zeinstra L, van den Born M, Korving J, Martens ACM, Barker N, van Oudenaarden A, Clevers H, Yee Nee AN, Gregorieff A, Sasaki N, Zeinstra L, van den Born M, Korving J, Martens ACM, Barker N, van Oudenaarden A, Clevers H. Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. *Nat Cell Biol*. 2012;14(10):1099-1104. doi:10.1038/ncb2581.
178. Nowotschin S, Xenopoulos P, Schrode N, Hadjantonakis A-K. A bright single-cell resolution live imaging reporter of Notch signaling in the mouse. *BMC Dev Biol*. 2013;13(1):15. doi:10.1186/1471-213X-13-15.

179. Vooijs M, Ong C-T, Hadland B, Huppert S, Liu Z, Korving J, van den Born M, Stappenbeck T, Wu Y, Clevers H, Kopan R. Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE. *Development*. 2007;134(3):535-544. doi:10.1242/dev.02733.
180. Surveillance, Epidemiology, and End Results Program. SEER Stat Fact Sheets: Stomach Cancer. National Cancer Institute.
181. Han M-E, Oh S-O. Gastric stem cells and gastric cancer stem cells. *Anat Cell Biol*. 2013;46:8-18. doi:10.5115/acb.2013.46.1.8.
182. Yeh TS, Wu CN, Hsu KW, Liao WJ, Yang MC, Li AFY, Wang AM, Kuo ML, Chi CW. The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res*. 2009;69(12):5039-5048. doi:10.1158/0008-5472.CAN-08-4021.
183. Sun Y, Gao X, Liu J, Kong QY, Wang XW, Chen XY, Wang Q, Cheng YF, Qu XX, Li H. Differential Notch1 and Notch2 expression and frequent activation of Notch signaling in gastric cancers. *Arch Pathol Lab Med*. 2011;135:451-458. doi:10.1043/2009-0665-OA.1.
184. Kang H, An JH, Song JY, Kim TH, Heo JH, Ahn DH, Kim G. NOTCH3 and JAGGED2 contribute to gastric cancer development and to glandular differentiation associated with MUC2 and MUC5AC expression. *Histopathology*. 2012;61:576-586. doi:10.1111/j.1365-2559.2012.04274.x.
185. Du X, Cheng Z, Wang Y-H, Guo Z-H, Zhang S-Q, Hu J-K, Zhou Z-G. Role of Notch signaling pathway in gastric cancer: A meta-analysis of the literature. *World J Gastroenterol*. 2014;20(27):9191-9199. doi:10.3748/wjg.v20.i27.9191.
186. Li G-G, Li L, Li C, Ye L-Y, Li X-W, Liu D-R, Bao Q, Zheng Y-X, Xiang D-P, Chen L, Chen J. Influence of up-regulation of Notch ligand DLL4 on biological behaviors of human gastric cancer cells. *World J Gastroenterol*. 2013;19(28):4486-4494. doi:10.3748/wjg.v19.i28.4486.
187. Hsu K-W, Hsieh R-H, Huang K-H, Fen-Yau Li A, Chi C-W, Wang T-Y, Tseng M-J, Wu K-J, Yeh T-S. Activation of the Notch1/STAT3/Twist signaling axis promotes gastric cancer progression. *Carcinogenesis*. 2012;33(8):1459-1467. doi:10.1093/carcin/bgs165.

188. Tseng Y-C, Tsai Y-H, Tseng M-J, Hsu K-W, Yang M-C, Huang K-H, Li AF-Y, Chi C-W, Hsieh R-H, Ku H-H, Yeh T-S. Notch2-induced COX-2 expression enhancing gastric cancer progression. *Mol Carcinog*. 2012;51(12):939-951. doi:10.1002/mc.20865.
189. Qian C, Liu F, Ye B, Zhang X, Liang Y, Yao J. Notch4 promotes gastric cancer growth through activation of Wnt1/ β -catenin signaling. *Mol Cell Biochem*. 2014;401:165-174. doi:10.1007/s11010-014-2304-z.
190. Piazzzi G, Fini L, Selgrad M, Garcia M, Daoud Y, Wex T, Malfertheiner P, Gasbarrini A, Romano M, Meyer RL, Genta RM, Fox JG, Boland CR, Bazzoli F, Ricciardiello L. Epigenetic regulation of Delta-Like1 controls Notch1 activation in gastric cancer. *Oncotarget*. 2011;2(12):1291-1301.

CHAPTER 2

NOTCH1 AND NOTCH2 RECEPTORS REGULATE MOUSE AND HUMAN GASTRIC ANTRAL EPITHELIAL CELL HOMEOSTASIS

***Note:** This chapter is based on a manuscript currently under revision for *Gut* (gutjnl-2015-310811)

2.1: SUMMARY

Objective

We tested the ability of Notch pathway receptors Notch1 and Notch2 to regulate stem and epithelial cell homeostasis in mouse and human gastric antral tissue.

Design

Mice were treated with the pan-Notch inhibitor dibenzazepine (DBZ) or inhibitory antibodies targeting Notch1 and/or Notch2. Epithelial proliferation, apoptosis and cellular differentiation were measured by histological and molecular approaches. Organoids were established from mouse and human antral glands; growth and differentiation were measured after treatment with Notch inhibitors.

Results

Notch1 and Notch2 are the predominant Notch receptors expressed in mouse and human antral tissue and organoid cultures. Combined inhibition of Notch1 and Notch2 in adult mice led to decreased epithelial cell proliferation, including reduced proliferation of LGR5 stem cells, and increased apoptosis, similar to the response to global Notch inhibition with DBZ. Less pronounced effects were observed after inhibition of individual receptors. Notch pathway inhibition with DBZ or combined inhibition of Notch1 and Notch2 led to increased differentiation of all gastric antral lineages, with remodeling of cells to express secretory products normally associated with other regions of the gastrointestinal tract, including intestine. Analysis of mouse and human organoids showed that Notch signaling through Notch1 and Notch2 is intrinsic to the epithelium and required for organoid growth.

Conclusions

Notch signaling is required to maintain gastric antral stem cells. Notch1 and Notch2 are the primary Notch receptors regulating epithelial cell homeostasis in both mouse and human stomach.

2.2: INTRODUCTION

The adult gastric epithelium is constantly renewed due to a population of actively cycling stem cells located in the gastric glands. These stem cells generate daughter cells that, upon exiting the stem cell niche, differentiate into the various epithelial cell lineages of the stomach. In the distal, antral stomach, active stem cells express the R-spondin receptor LGR5, which also marks stem cells in the intestine and other tissues^{1,2}. Antral LGR5 stem cells give rise to all antral lineages, including surface

mucous cells, endocrine cells and deep mucous cells. The signaling pathways regulating gastric stem cell proliferation and differentiation are currently poorly understood.

Notch signaling is well described to maintain intestinal stem cells³⁻⁷ and recent studies suggest that gastric stem cells are similarly regulated by Notch^{8,9}. In the stomach, pan-Notch inhibition led to reduced gastric stem and epithelial cell proliferation and increased differentiation of mucous and endocrine cell lineages. In contrast, activation of Notch through constitutive expression of the Notch Intracellular Domain (NICD) induce stem cell proliferation, gland fission and ultimately hyper-proliferative polyps^{8,9}. Furthermore, increased expression of Notch signaling components has been associated with gastric cancer, suggesting Notch pathway involvement^{10,11}.

Four Notch receptors (Notch1-4) exist in vertebrates that are single-pass transmembrane proteins¹². Receptor signaling involves proteolytic receptor cleavage to release the intracellular signaling component NICD, which activates target gene transcription, such as those in the *Hes* and *Hey* families¹³. Notch1 and Notch2 are the primary receptors involved in intestinal stem cell homeostasis, with Notch1 having a predominant function^{5,7,14,15}. Global pharmacological Notch inhibition leads to intestinal toxicity³, but inhibition of Notch1 alone revealed a partial Notch-inhibition phenotype while avoiding major toxicity^{7,14,15}.

The specific Notch receptors regulating the stomach have not been described. In this study we examined the role of Notch receptors in epithelial and LGR5 stem cell homeostasis in the gastric antrum of genetic mouse models. We find that Notch1 and Notch2 are key regulators of stem cell proliferation, differentiation and apoptosis.

Furthermore our studies demonstrate that both Notch1 and Notch2 function to regulate growth of antral organoid cultures generated from human and mouse tissue.

2.3: METHODS

Mice

Mice of both sexes aged 2-3 months were used. *Lgr5-EGFP-IRES-CreERT2* (*Lgr5-GFP*)² (Jackson Labs #008875), *Notch1-CreERT2^{SAT}* (*N1Cre*) and *Notch2-CreERT2^{SAT}* (*N2Cre*)¹⁶, *CBF:H2B-Venus*¹⁷ (Jackson Labs #020942) and *ROSA26-CAG-LSL-tdTomato-WPRE* (*ROSA-Tom*)¹⁸ (Jackson Labs #007909) mice were previously described. All mice were on a C57BL/6 background except for *CBF:H2B-Venus*, which was on a mixed background (CD1 and FVB/N). Mice were housed under specific pathogen-free conditions in automated watered and ventilated cages on a 12-hour light/dark cycle. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals. For lineage tracing Notch1- or Notch2-expressing cells, *N1Cre*; *ROSA-Tom* and *N2Cre*; *ROSA-Tom* mice were treated with either 1 injection of tamoxifen (1mg/20g body weight) followed by a 3-day chase or 5 daily injections of tamoxifen followed by a 2-week chase.

Notch Pathway Inhibition

For *in vivo* Notch inhibition, the gamma-secretase inhibitor dibenzazepine (DBZ, 30µmol/kg i.p., SYNCOM, Groningen, The Netherlands) or vehicle (0.1% Tween-80, 0.5% hydroxypropylmethylcellulose [E4M], 0.1% DMSO in water) was administered to *Lgr5-GFP* mice once per day for 5 days, with tissue collected the sixth day. Humanized

IgG1 neutralizing monoclonal antibodies specific for the Notch1- or Notch2-negative regulatory region (α N1 or α N2), or an irrelevant control IgG1 antibody interacting with herpes simplex virus gD protein (α Gd) were described previously¹⁵. Antibodies were injected i.p. at 5mg/kg on days 1 and 4, with collection of stomach tissue on day 6, 14, or 28.

For *in vitro* treatment of mouse and human organoids, the gamma-secretase inhibitor *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester (DAPT, 1 μ M; EMD4Biosciences, Gibbstown, NJ, USA), α Gd, α N1 or α N2 (10 μ g/mL) were added to culture media and renewed every other day for 5 days in established organoid lines.

Tissue Collection and Histological Analysis

Mice were fasted overnight with free access to water before tissue collection. For some experiments, mice were injected with 5-ethynyl-2'-deoxyuridine (EdU, 25 mg/kg, Invitrogen, Grand Island, NY, USA) 1.5 hr prior to tissue collection. Stomachs were processed for cryo and paraffin histology as described⁹. For analysis of proliferation, EdU incorporation was visualized with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies, Carlsbad, CA, USA). Tissue sections were incubated with primary antibodies (Supplementary Table 2-1) followed by appropriate secondary antibodies (1:400, Invitrogen) and mounted with ProLong Gold containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) as described previously¹⁹. To analyze proliferating LGR5 stem cells, sections were immunostained for GFP (chicken) and Ki67 as described⁹. Antral glands were isolated and immunostained as previously

described,⁹. Imaging by digital microscopy was done using a Leica SP5X Inverted 2-Photon FLIM Confocal Microscope or a Nikon E-800 Microscope.

For ultrastructural analysis, tissue was fixed and Epon embedded as described⁶. Electron micrographs were captured using a JEOL JEM 1400 plus electron microscope.

Gene Expression Analysis

RNA was isolated from antral tissue or gastric organoids as previously described⁹. RNA was isolated from human antral tissue using Trizol (Invitrogen), followed by DNase treatment and purification using the RNeasy Mini Kit (Qiagen). cDNA was prepared from 500ng total RNA and quantitative RT-PCR was performed as described²⁰ using primers listed in Supplemental Table 2-2, normalized to *Gapdh* (mouse) or *ACTB* (human).

Gastric Organoid Culture

Mouse gastric organoid culture was carried out as previously described^{9,21}. In brief, antral glands were resuspended in Matrigel (Corning, Tewksbury, MA, USA) and overlaid with culture media (50% L-WRN conditioned media, Advanced DMEM/F12, 10% FBS, 1X Pen-Strep, 2 mM L-Glutamine), with the addition of Y-27632 (10 μ M, Tocris, Bristol, United Kingdom) only upon initial plating. L-WRN conditioned media containing Wnt3a, R-spondin3, and Noggin was generated as described²¹. Human antral tissue (surgical resection or Gift of Life) was obtained under Institutional Review Board approved protocols and organoids were established as described²² with modifications. Tissue was incubated with 10mM dithiothreitol (DTT; Invitrogen) in DPBS

with Pen-Strep and Gentamycin for 15 min at room temperature, followed by incubation in 12mM EDTA in DPBS with Pen-Strep and Gentamycin for 1 hr at 4°C on a rocking platform, vigorously shaken for 1 min to release glands and pelleted at 100 g for 5 min. Glands were resuspended in Matrigel and after 30 min at 37°C, culture media described above with the addition of Y-27632 and SB431542 (10 µM, Tocris Bioscience) was added to each well. Media was renewed every other day. Studies were performed in 3 independent established organoid lines that had been passaged at least 3 times before analysis.

Morphometrics

Morphometric analysis used ImageJ software (1.46r, Wayne Rasband, NIH, USA). For EdU incorporation, cleaved caspase-3 cells and gastrin cells, the entire length of the antrum for each animal was imaged (n=4-6 animals per group) and cell counts were normalized to epithelial length (µm). For LGR5 stem cell proliferation, the number of GFP/Ki67 double-positive cells per gastric antral gland was counted in αGd control (n=833 glands), αN1 (n=674 glands), αN2 (n=662 glands) and αN1+αN2 (n=548 glands) (n=5-7 mice/group). For organoid measurements, the area of at least 300 organoids per treatment was measured and data are presented as fold change compared to control (vehicle or αGd) organoids.

Statistics

GraphPad Prism software was used for statistical analysis. Quantitative data are presented as mean ± SEM and analyzed using Student's t-test (comparing DBZ/DAPT

to vehicle) or 1-way ANOVA with Dunnett's or Tukey's *post-hoc* test (comparing α N1, α N2, α N1+ α N2 and α Gd). qRT-PCR data are expressed as mRNA fold-change vs. control (vehicle or α Gd) with $P < 0.05$ considered significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

2.4: RESULTS

Notch signaling in antral stem cells

We used the *CBF:H2B-Venus* reporter mouse to identify cells undergoing Notch signaling in the gastric antrum. This transgenic mouse expresses a nuclear fluorescent protein in response to Notch receptor activation¹⁷. Isolated antral glands showed scattered Venus-positive cells that were primarily located in the gland base (Fig 2-1A). Costaining showed Venus expression in differentiated cell types, including CHGA-expressing enteroendocrine cells (Fig 2-1B) and DCLK1-expressing tuft cells (Fig 2-1C). Crossing the Notch reporter strain to *Lgr5-GFP* mice showed that some of the nuclear Venus-positive cells were also marked with cytoplasmic GFP, indicating Notch signaling in antral LGR5 stem cells (Fig 2-1D).

Gene expression analysis for the four Notch receptors showed that Notch1 and Notch2 were the primary Notch receptors expressed in full thickness antral tissue and isolated glands (Fig 2-1E, Supplementary Fig 2-1). To test if Notch receptors are present in antral stem cells, we performed a lineage tracing experiment with mice expressing Notch receptor-CreERT2 genes crossed to ROSA-Tom mice. We examined *N1Cre;ROSA-Tom* and *N2Cre;ROSA-Tom* mice 3 days and 2 weeks post-tamoxifen treatment. Tomato-marked epithelial cells were evident in the antral gland base of *N1Cre;ROSA-Tom* and *N2Cre;ROSA-Tom* mice at 3-days post induction (Fig 2-1F, arrows). At 2 weeks post-tamoxifen epithelial lineage stripes were detected in the antrum of *N1Cre;ROSA-Tom* mice (arrows), suggesting that Notch1 is expressed in antral stem cells. The lack of lineage stripes in *N2Cre;ROSA-Tom* mice suggests that this receptor is expressed in short-lived cells.

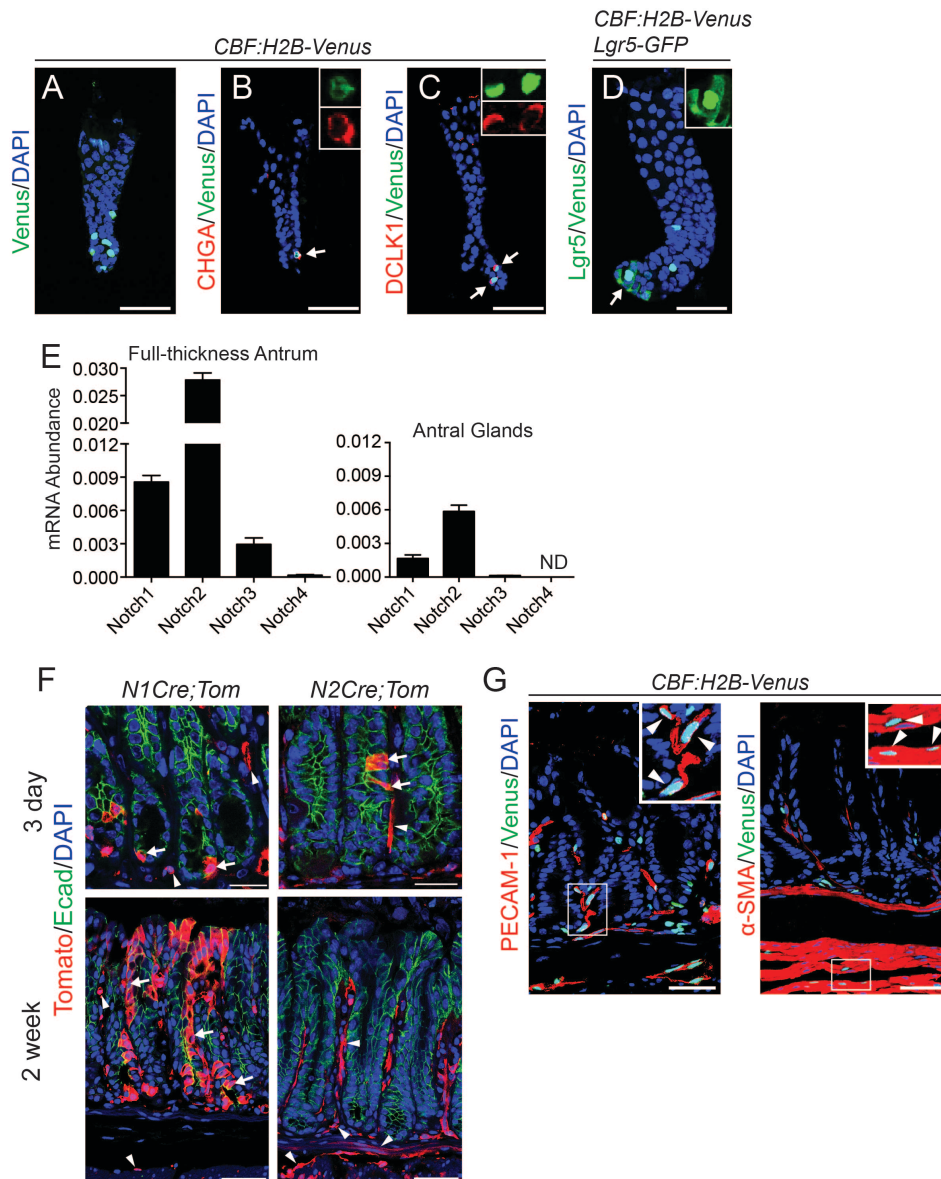


Figure 2-1. Notch signaling in gastric antral cells. (A-D) Antral glands isolated from *CBF:H2B-Venus* mice (green, nuclear) co-stained for (B) chromogranin A (CHGA) (red), (C) doublecortin like kinase 1 (DCLK1) (red), or (D) Lgr5-GFP (green, cytoplasmic) and DAPI (blue). Insets show higher-powered views for each channel. (E) Notch receptor expression was determined by qRT-PCR analysis of total RNA isolated from full-thickness antral tissue or antral glands (mean \pm SEM; n=3-5 mice). ND = Not detected. (F) Confocal imaging of paraffin sections from *N1-Cre;Tom* and *N2-Cre;Tom* mice co-stained for RFP (red), E-cadherin (green) and DAPI (blue) 3 days or 2 weeks after tamoxifen activation of Cre recombinase. Arrows and arrowheads indicate epithelial and non-epithelial Tomato expression, respectively. (G) Frozen tissue sections from *CBF:H2B-Venus* mice co-stained for the endothelial marker PECAM-1 (left, red) or the smooth muscle cell marker α -SMA (right, red). Co-stained cells in boxed regions are marked (arrowheads) in higher-powered insets. Scale bar: 50 μ m. (ED performed A-D, NLC performed G)

In addition to epithelial cell labeling there was extensive non-epithelial Notch lineage marking with both *N1Cre;ROSA-Tom* and *N2Cre;ROSA-Tom* (Fig 2-1F, arrowheads). Histological analysis of tissue sections from the *CBF:H2B-Venus* reporter mouse demonstrated that the majority of Venus-positive stromal cells are PECAM-1-expressing endothelial cells and α -smooth muscle actin-expressing smooth muscle cells (Fig 2-1G).

Notch regulation of LGR5 stem cells

To test the function of Notch1 and Notch2 to regulate antral epithelial cell homeostasis we first examined proliferation after treatment with either inhibitory antibodies that selectively target Notch1 and Notch2 or the gamma-secretase inhibitor DBZ (a pan-Notch inhibitor). Analysis of EdU incorporation showed that cellular proliferation was reduced with combined α N1+ α N2 treatment to a similar extent as DBZ (Fig 2-2A-H). Treatment with α N1 or α N2 alone significantly reduced the number of proliferating cells, but to a lesser extent than combined receptor blockade (Fig. 2-2D-H). Analysis at 2 and 4 weeks after α N1 or α N2 treatment demonstrated normal proliferation after the antibody treatment was discontinued (Fig 2-2H). However, morbidity of the α N1+ α N2 group prevented analysis of this group at these later time points.

To directly investigate LGR5 stem cells, we treated *Lgr5-GFP* mice with α N1 and/or α N2 and analyzed stem cell proliferation via co-immunostaining for GFP and Ki67 (Fig 2-2I, arrow). Notch1 and/or Notch2 inhibition reduced LGR5 stem cell proliferation, with α N1+ α N2 having the most substantial effect (Fig 2-2J), which was

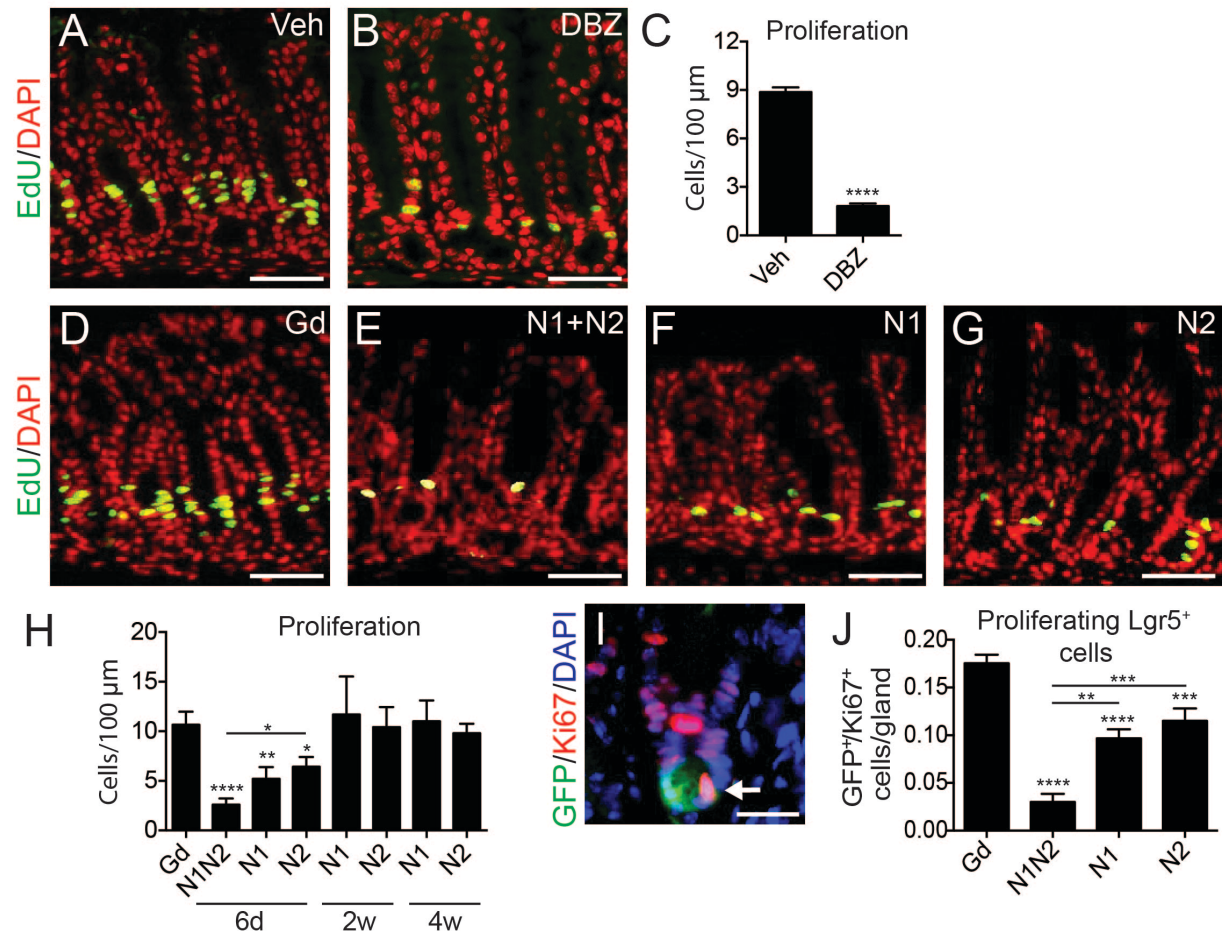


Figure 2-2. Notch1 and Notch2 regulate antral cell proliferation. (A,B,D-G) Proliferating cells were detected in (A) vehicle, (B) DBZ, (D) Gd antibody control (α Gd), (E) α N1+ α N2, (F) α N1, and (G) α N2 tissues by staining for EdU (green) with DAPI (red). (C, H) Morphometric quantification of EdU cells (mean \pm SEM; n=5-7 mice). (I, J) Proliferating LGR5 stem cells were visualized in treated Lgr5-GFP mice by GFP (green) and Ki67 (red) co-staining (arrow) with DAPI (blue) and quantified by morphometrics (mean \pm SEM; n=5-7 mice; 40-100 glands/mouse). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. vehicle using Student's t-test or vs. α Gd using 1-way ANOVA. Scale bar: 50 μ m (A,B,D-G) or 25 μ m (I).

similar to our previous analysis of pan-Notch inhibition with DBZ⁹. The similarity of combined N1 and N2 receptor blockade to pan-Notch inhibition suggests that Notch1 and Notch2 are the key receptors mediating Notch effects on stem cell proliferation.

We next analyzed cell death by staining for the apoptotic marker cleaved caspase 3. Apoptotic cells are normally rare in the antral epithelium. Notch inhibition with DBZ or receptor blockade was observed to increase the number of caspase-positive cells, with apoptotic cells predominantly localized in the gland base (Fig 2-3). As with the proliferation findings, α N1+ α N2 treatment was comparable to DBZ, with more modest effects observed after treatment with antibodies targeting individual receptors (Fig 2-3C-D).

Notch regulates antral organoid growth

We established organoids to test whether the Notch effects are intrinsic to the epithelial cells. Organoids established from the *CBF:H2B-Venus* reporter mouse showed extensive Venus labeling, demonstrating active Notch signaling in organoid culture (Fig 2-4A). Gene expression analysis showed that, similar to what was observed in gastric glands, Notch1 and Notch2 were the primary Notch receptors expressed in antral organoids (Fig 2-4B). To investigate Notch function we treated organoids with the pan-Notch inhibitor DAPT and observed reduced overall growth (Fig 2-4C-E). Reduced growth was also observed after receptor targeting, with combined N1+N2 inhibition similar to pan-Notch inhibition, and individual receptor targeting showing a larger role for Notch1 to regulate growth (Fig 2-4F-J). These findings demonstrate that intrinsic Notch signaling is required for antral organoid growth.

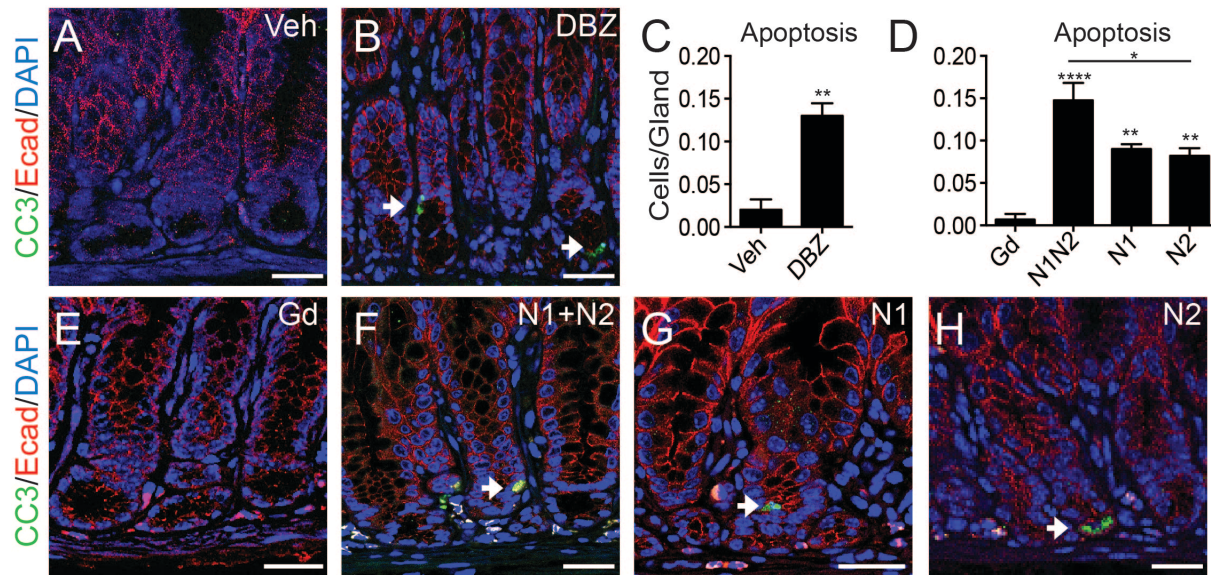


Figure 2-3. Enhanced apoptosis after Notch inhibition. (A,B, E-H) Apoptosis was measured by co-staining for cleaved caspase-3 (CC3) (green) and E-cadherin (Ecad) (red) with DAPI (blue) in (A) vehicle, (B) DBZ, (E) α Gd, (F) α N1+ α N2, (G) α N1, and (H) α N2-treated mice. (C,D) Morphometric quantification of apoptotic CC3/E-cad co-stained cells (arrows) (mean \pm SEM; n=5-7 mice). Scale bar: 20 μ m.

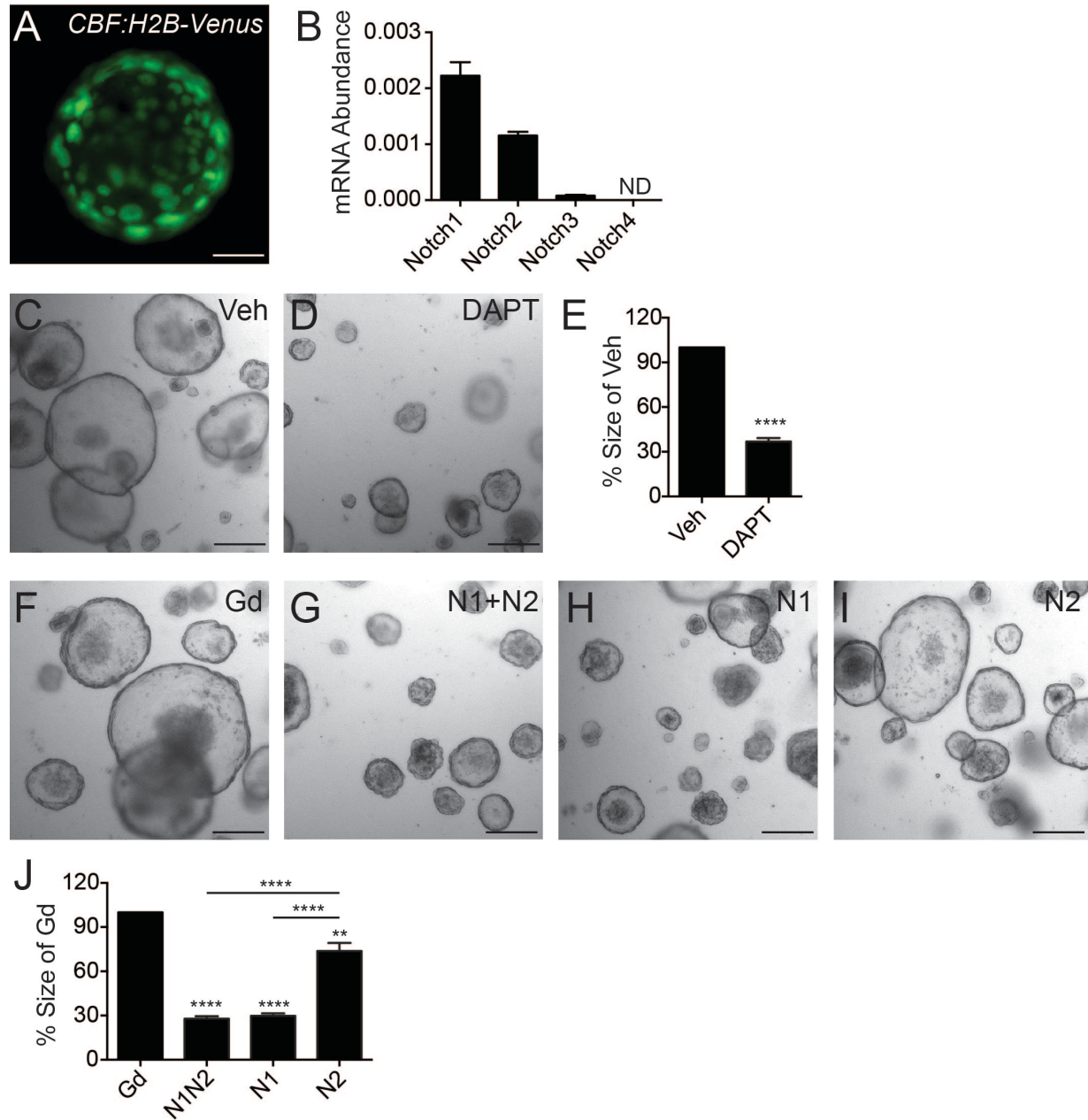


Figure 2-4. Notch1 and Notch2 regulate mouse antral organoid growth. (A) Cells undergoing Notch signaling (green) were observed in antral organoids established from the *CBF:H2B-Venus* Notch reporter mouse strain. (B) Gene expression of Notch receptors determined by qRT-PCR analysis of antral organoids (mean \pm SEM n=3 independent organoid lines). ND = Not detected. Morphology (C,D,F-I) and quantitative measure of organoid size (E,J) following 5 days of treatment with (C) vehicle, (D) DAPT, (F) α Gd, (G) α N1+ α N2, (H) α N1, or (I) α N2 (mean \pm SEM; n=3 lines). Scale bar: 50 μ m (A) or 250 μ m (C,D,F-I).

Notch1 and Notch2 regulate antral epithelial cell differentiation

We previously showed that Notch inhibition was associated with increased antral cell differentiation⁹. Thus, we analyzed the role of Notch1 and Notch2 for the major antral cell lineages: enteroendocrine, surface mucous and deep mucous cells. Analysis of enteroendocrine cells showed increased numbers of gastrin-expressing cells and increased abundance of *Gast* and *Chga* mRNAs with Notch inhibition (Fig 2-5). Similar effects were observed after treatment with DBZ (Fig 2-5A-E) and α N1+ α N2, but not with α N1 or α N2 treatment, suggesting that these two receptors are fully redundant for this function (Fig 2-5F-L). In accordance with the *in vivo* findings, increased *Gast* mRNA was observed in organoids after DAPT or α N1+ α N2 treatment (Fig 2-5M,N).

Similarly, analysis of the surface mucous cell marker CLCA1 showed expansion of this cell population after DBZ (Fig 2-6A-C) or combined α N1+ α N2 treatment, but not after individual receptor blockade (Fig 2-6D-H). Consistent with the marker expression data, ultrastructural analysis showed a marked expansion of surface mucous cells (Supplementary Fig 2-2). Electron microscopy demonstrated increased numbers of granules in these cells after DBZ treatment (Fig 2-6I,J).

Analysis of H&E stained tissues suggested increased mucous cells at the gland base with DBZ or inhibition of both receptors (Supplementary Fig 2-3). Accordingly, GSII-lectin staining showed an apparent expansion of this cell lineage after DBZ or α N1+ α N2 treatment (Fig 2-7A,B,E-H), which was confirmed by increased trefoil factor 2 (*Tff2*) mRNA abundance (Fig 2-7C,I). Surprisingly, further analysis of these cells also showed increased staining for the chief cell marker gastric intrinsic factor (GIF), which was confirmed by increased *Gif* mRNA (Fig 2-7D,J). High-powered confocal analysis

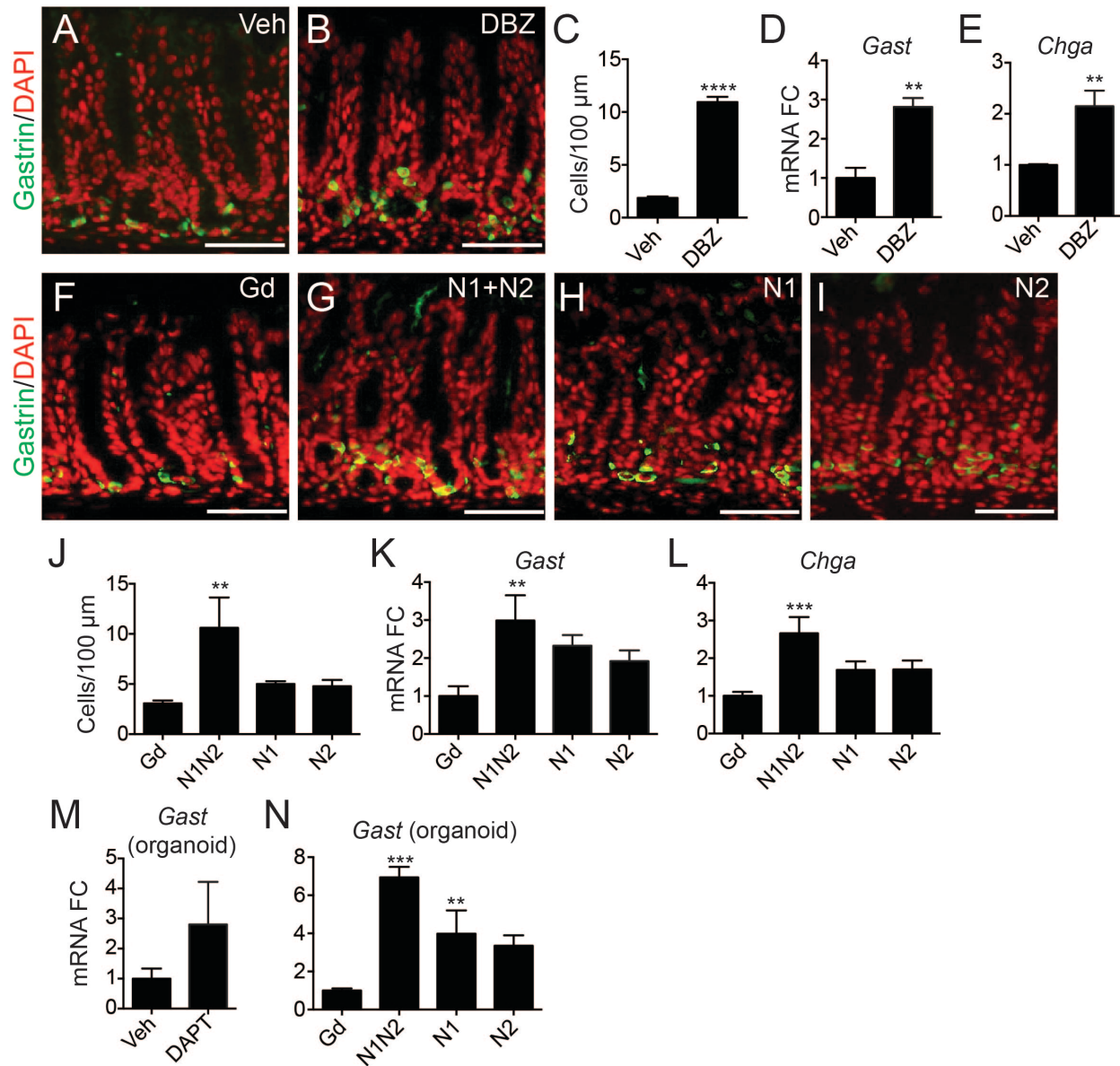


Figure 2-5. Notch1 and Notch2 regulate antral endocrine cell differentiation. (A,B, F-I) Gastrin immunostaining (green) of paraffin sections from mice treated with (A) vehicle, (B) DBZ, (F) α Gd, (G) α N1+ α N2, (H) α N1, and (I) α N2 with DAPI (red). (C,J) Gastrin cells were quantitated by morphometrics (mean \pm SEM, n=4-5). Gene expression analysis of gastrin (*Gast*) and chromogranin A (*Chga*) in (D,E,K,L) full-thickness tissue or (M,N) organoids (mean \pm SEM; n=5-7 mice or 3 independent organoid lines). Scale bar: 50 μ m.

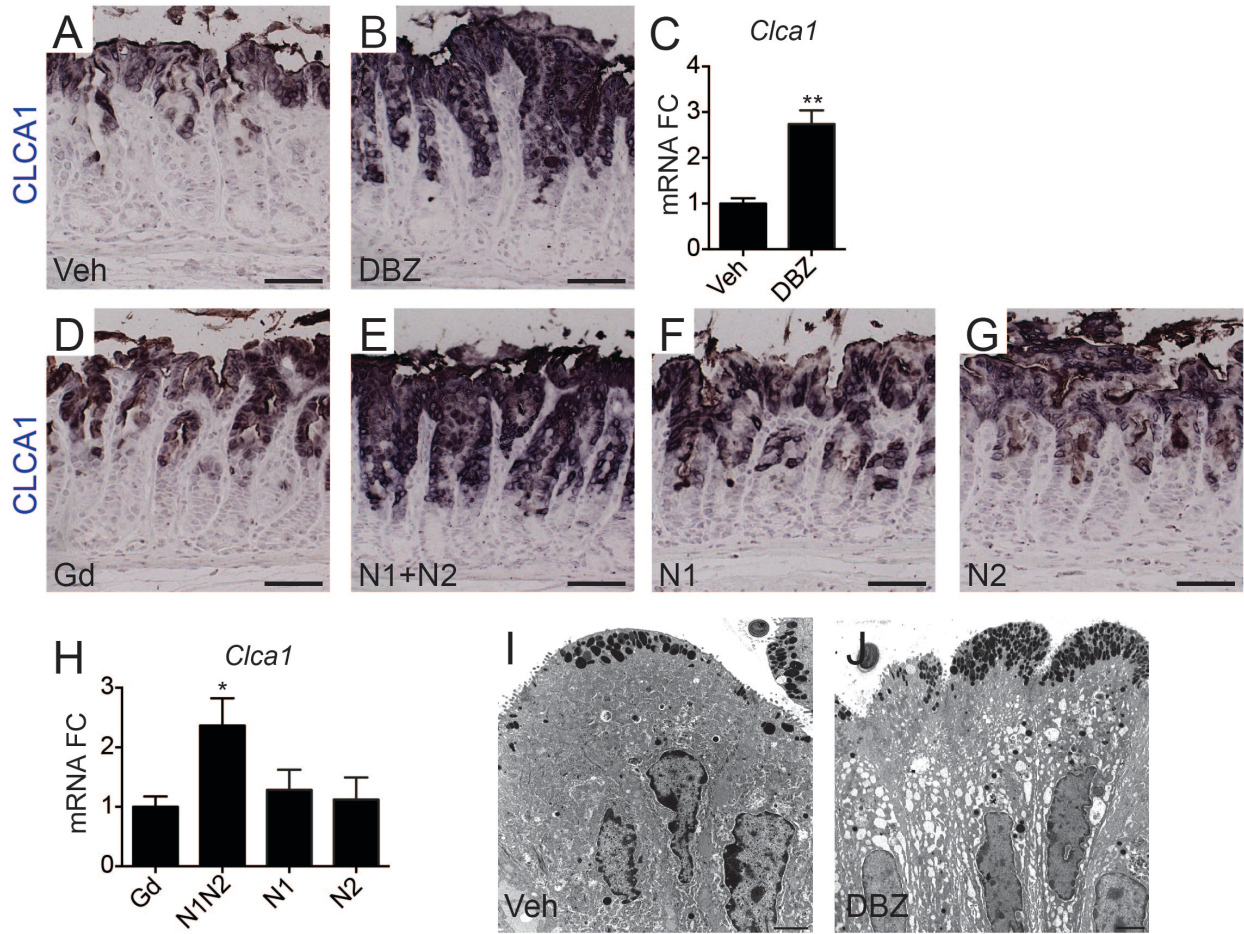


Figure 2-6. Notch1 and Notch2 regulate surface mucous cell differentiation. (A, B, D-G) Immunostaining for the surface mucous cell marker CLCA1 in (A) vehicle, (B) DBZ, (D) α Gd, (E) α N1+ α N2, (F) α N1, and (G) α N2-treated mice. (C,H) qRT-PCR analysis of *Clca1* gene expression in antral tissue (mean \pm SEM; n=5-7 mice). (I,J) Transmission electron microscope images of surface mucous cells from (I) vehicle or (J) DBZ-treated mice. Scale bar: 50 μ m (A,B,D-G) or 2 μ m (I,J).

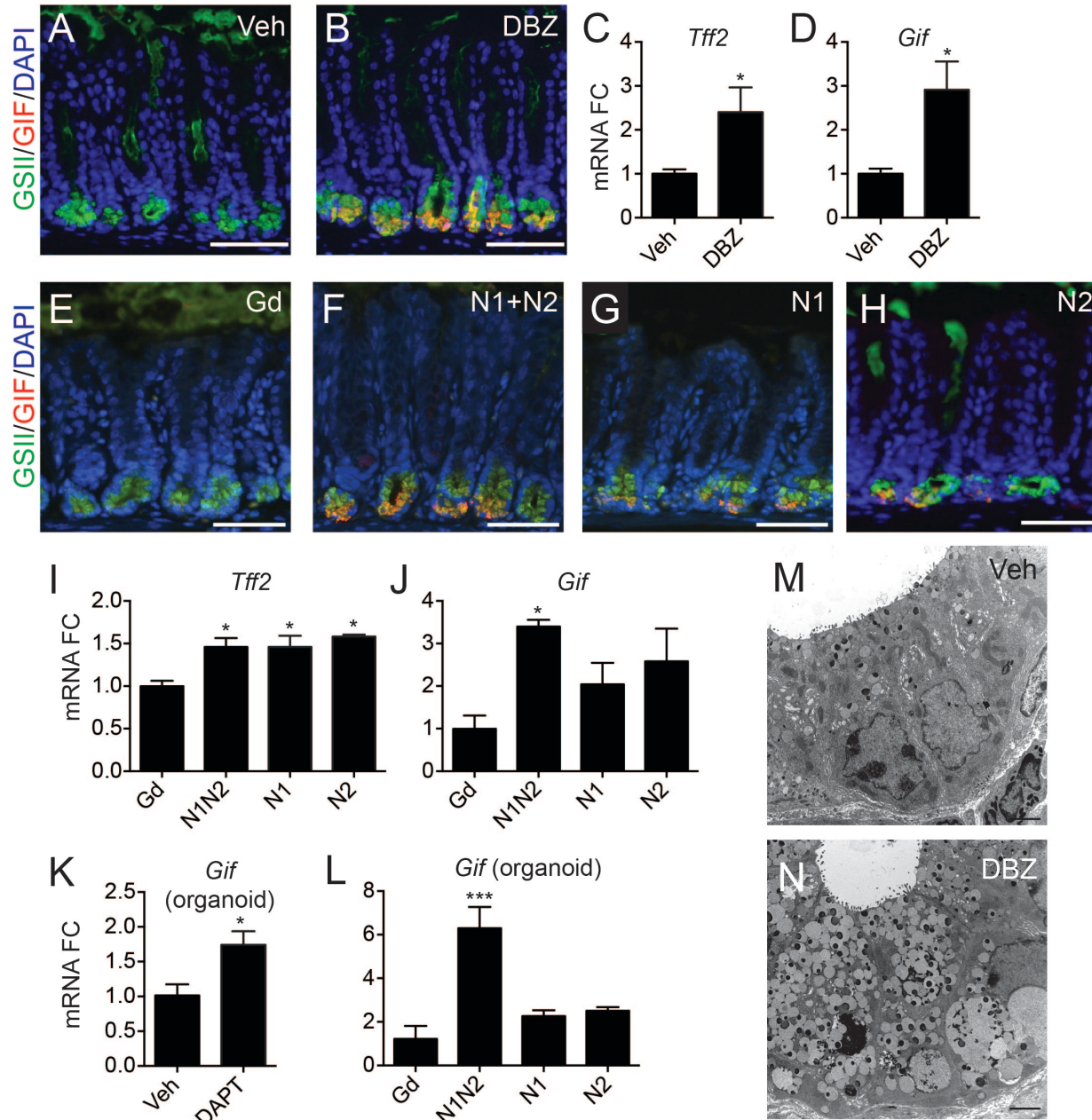


Figure 2-7. Notch inhibition remodels cells at the antral gland base. (A,B, E-H) Co-staining with GSII lectin (green) and an anti-gastric intrinsic factor (GIF) antibody (red) with DAPI (blue) on (A) vehicle, (B) DBZ, (E) α Gd, (F) α N1+ α N2, (G) α N1, and (H) α N2-treated mice. (C,D I-L) Gene expression analysis of trefoil factor 2 (*Tff2*) and gastric intrinsic factor (*Gif*) by qRT-PCR analysis of full-thickness tissue or organoids (mean \pm SEM; n=4-7 mice). (M,N) Transmission electron microscope images of gland base cells from (M) vehicle or (N) DBZ-treated mice. Scale bar: 50 μ m (A,B,E-H) or 2 μ m (M,N).

showed GSII and GIF co-labeled cells at the gland base, primarily localized in separate granules (Supplementary Fig 2-4). Ultrastructural analysis showed a marked increase in both secretory granule number and granule size in cells at the gland base after Notch inhibition (Fig 2-7M,N and Supplementary Fig 2-2). Consistent with the *in vivo* data, increased *Gif* expression was also observed in DAPT or α N1+ α N2 treated antral organoids (Fig 2-7K,L).

To better understand the cellular remodeling observed in the Notch-inhibited antrum we examined markers associated with the gastric corpus and intestine. Increased immunostaining and gene expression was observed for the corpus ECL cell marker histidine decarboxylase (HDC; Fig 2-8A-C), however there was no change in expression of the corpus parietal cell marker H,K-ATPase (data not shown). We also observed increased expression of markers of intestinal cells, including goblet cells (*Muc2* and *Tff3*) and Paneth cells (*Mmp7*, *Cryptdin*, *Reg3 γ*) in the DBZ-treated antrum (Fig 2-8D-J). However, similar to the corpus markers, the increase in intestinal markers was not associated with a general intestinalization as the DBZ-treated antrum did not increase expression of the intestinal markers *Cdx2*, *Vil1*, *Cck* or *Lyz1* (data not shown) and continued to express gastric specific markers (*Tff2* and *Gif*) and

Together these findings suggest that global Notch inhibition with DBZ or combined α N1+ α N2 treatment results in a generalized increase in cellular differentiation. However, dual receptor blockade is required for the differentiation effect, suggesting that Notch1 and Notch2 function together during differentiation.

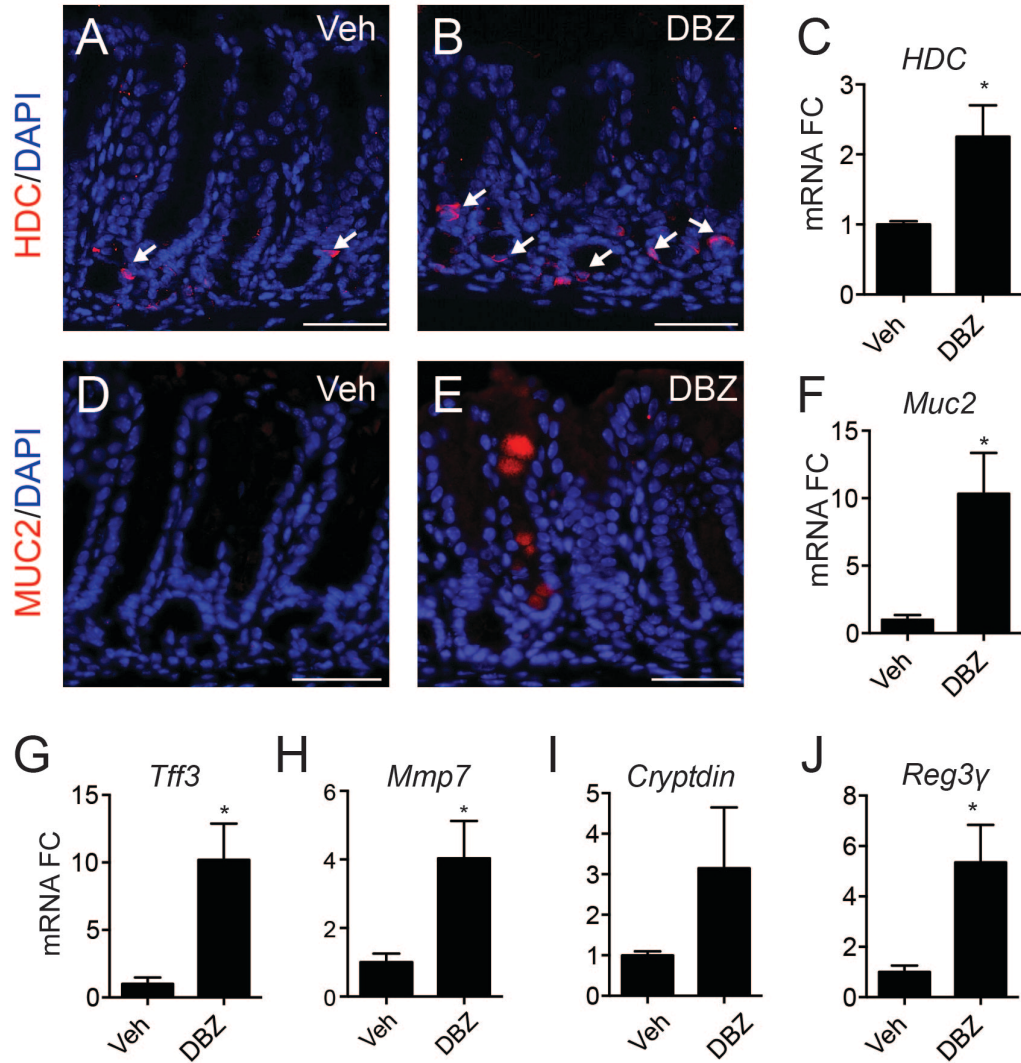


Figure 2-8. Increased expression of gastric corpus and intestinal cell markers in DBZ-treated antral tissue. (A-C) Immunostaining and gene expression analysis of histidine decarboxylase (HDC). (D-F) Immunostaining and gene expression analysis of Muc2. (G-J) Gene expression analysis of trefoil factor 3 (*Tff3*), matrix metalloproteinase-7 (*Mmp7*), *cryptdin*, and regenerating islet-derived 3 gamma (*Reg3γ*) in antral RNA isolated from vehicle or DBZ-treated mice (mean \pm SEM; n=3-6 mice).

Notch is necessary for growth of human gastric organoids

We took advantage of the design feature of the inhibitory antibodies to target both mouse and human Notch1 and Notch2 to test the regulation of human gastric stem cells *in vitro* in organoids derived from human gastric glands. We first determined which Notch receptors were expressed by qRT-PCR, showing that similar to our findings in mouse, Notch1 and Notch2 were the predominant receptors expressed in human antral tissue and organoids (Fig 2-9A,B and Supplementary Fig 2-5,2-6, Supplementary Table 2-3).

We confirmed that intrinsic Notch signaling was required for growth of human organoids, with reduced organoid size observed after addition of DAPT to the culture media (Fig 2-9C-E). Treatment with α N1+ α N2, α N1 or α N2 also reduced growth, with a pattern remarkably similar to what we observed in mouse organoids. These findings suggest that Notch1 and Notch2 together regulate human antral organoid growth and that the mouse serves as a valid model to study human gastric epithelial stem cell function.

2.5: DISCUSSION

Here we report that Notch signaling regulates gastric antral epithelial cell homeostasis through the Notch1 and Notch2 receptors. Inhibition of both Notch1 and Notch2 signaling mimics effects on proliferation and differentiation observed with global Notch inhibition with DBZ treatment. Notch signaling in antral LGR5 stem cells was shown by Notch reporter expression and lineage tracing studies. We also showed that Notch signaling is crucial for proliferation of gastric LGR5 stem cells, with both Notch1

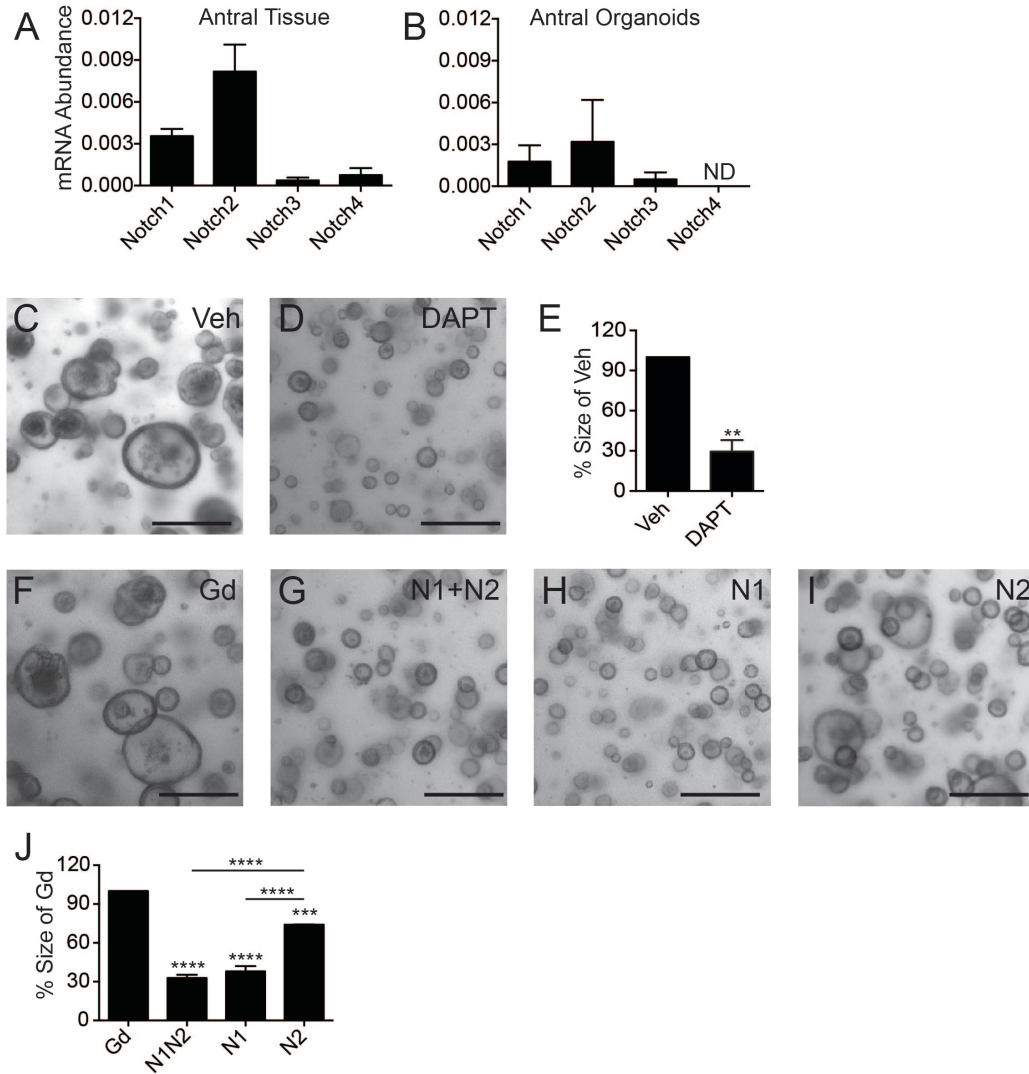


Figure 2-9. Human antral organoid growth is regulated by Notch1 and Notch2. (A, B) Notch receptor mRNA abundance was measured by qRT-PCR in (A) full-thickness human tissue or (B) antral organoids (mean \pm SEM, n=3 patients or patient-derived organoid lines). See Supplementary Figures 2-5 and 2-6, and Supplementary Table 2-3 for detailed information on human tissues. Morphology (C,D,F-I) and quantitative measure of organoid size (E,J) following treatment with (C) vehicle, (D) DAPT, (F) α Gd, (G) α N1+ α N2, (H) α N1, or (I) α N2 (mean \pm SEM, n=3 independent patient-derived organoid lines). Scale bar: 250 μ m. ND = Not detected.

and Notch2 functioning to promote stem cell proliferation. Our findings suggest that Notch1 is the primary receptor in LGR5 stem cells as we observed full lineage stripes with *N1Cre; ROSA-Tom* mice but not with *N2Cre; ROSA-Tom* mice. In addition α N1 was more effective than α N2 in inhibiting organoid growth. Further studies will be needed to understand how Notch1 and Notch2 regulate transit amplifying progenitors versus stem cells.

Our finding that Notch signaling stimulates gastric stem and progenitor cell proliferation is consistent with previous reports associating Notch pathway activation with gastric tumorigenesis. Upregulation of Notch pathway components has been reported in human gastric cancer, including Notch1, Notch2, Dll4, and Hes1^{10,11,23}, with Notch1 being associated with diffuse type and Jagged1 being overexpressed in both diffuse and poorly differentiated type cancers²⁴. Consistent with a potential role for Notch in promoting gastric tumorigenesis, genetic mouse models of Notch overexpression in the stomach induced hyperplastic polyps^{8,9}.

We further demonstrate that Notch1 and Notch2 regulate epithelial cell differentiation. Inhibition of both Notch1 and Notch2 signaling resulted in significant increases in all differentiated lineages, with increases in endocrine, surface mucous and deep mucous cells, similar to what is seen with global Notch inhibition. Our observation that individual inhibition of Notch1 or Notch2 did not broadly change differentiated cell types suggests that these receptors generally have redundant roles in differentiation. Notch regulation of differentiation in the stomach differs from the action of Notch in the intestine, where it has been well established that pathway inhibition induces a cell fate switch leading to secretory cell hyperplasia^{3,4,6}. Thus, while Notch signaling in the

intestine promotes enterocyte differentiation, Notch signaling in the stomach appears to generally repress differentiation of all lineages⁹. It is important to note however that in contrast to the intestine, the adult stomach only contains secretory cells.

Interestingly, we observed that Notch inhibition resulted in marked cellular remodeling. Cells at the surface exhibited an increased number of secretory granules to accompany the observed increases in immunostaining and gene expression of surface mucous cell markers. Tissue remodeling in the gland base was characterized by increased density and size of secretory granules and upregulation of intrinsic factor immunostaining. We also observed an increase in expression of selected intestinal and corpus cell markers, although the expression pattern did not suggest conversion of the antrum to these other gastrointestinal tissues. Further studies are needed to understand the role Notch plays in maintaining antral cell regional identity.

Cells that co-express intrinsic factor and mucous cell markers, such as TFF2, have been previously described in both immature and adult stomach. In mouse, co-expressing cells are observed throughout the immature newborn stomach, including the proximal corpus and distal antral regions²⁰. Moreover, in adult human and mouse stomach, co-expressing cells have been observed in adult corpus as a metaplastic cell lineage termed spasmolytic polypeptide-expressing metaplasia (SPEM)²⁵. SPEM is induced in response to loss of parietal cells and has been proposed to be a precursor to gastric cancer²⁶⁻²⁸. How the remodeled antral cells in the Notch-inhibited antrum relate to these previously described cell types will be an interesting future question.

The development of methods to grow epithelial organoids from primary mouse and human tissue is a powerful approach to study antral stem cells^{1,22,29,30}. Either

complete Notch blockade with GSI, or specific receptor targeting with α N1 or α N2 inhibitory antibodies had similar effects of reducing overall organoid growth, with Notch1 playing a more significant role than Notch2. This finding agrees with our data showing active Notch1 signaling in antral LGR5 stem cells. Furthermore these studies showed that Notch signaling is intrinsic to the gastric epithelium, with signaling required for organoid growth in culture. In addition, we observed that Notch regulates organoid differentiation, with upregulation of zymogenic and endocrine cell marker expression after Notch inhibition with DAPT or combined α N1+ α N2 treatment, similar to what we observed *in vivo*.

Our finding that Notch1 and Notch2 receptor signaling regulates human stomach epithelium has therapeutic implications. Notch pathway dysregulation is associated with the development of several cancers and thus this pathway is a prime target of therapeutic significance³¹. The use of pan-Notch inhibitors such as GSIs for Notch-related pathologies has been of clinical relevance for years; however gastrointestinal toxicity severely limits their use³. Thus more specific pathway targets are being explored. In particular, the development of humanized inhibitory antibodies against either Notch1 or Notch2 might allow for dose-dependent inhibition of each receptor while avoiding the side effects seen with global Notch inhibition¹⁵. However, our findings predict that targeting Notch1 or Notch2 to treat cancer might be associated with disruption of gastric epithelial cell homeostasis and thus gastric cellular changes should be considered in patients undergoing targeted Notch inhibition.

2.6: ACKNOWLEDGMENTS AND FUNDING

We thank Dr. Thaddeus Stappenbeck for L-WRN cells, Dr. John Williams and Bradley Nelson for assistance with electron microscopy, Dr. Spyros Artavanis-Tsakonas for use of the N1Cre and N2Cre mice and Christopher Altheim for generating L-WRN conditioned media. GBG was supported by NIH T-32-GM0008322 and NIH T32-DK094775 , ESD was supported by NIH F32-DK093349, and ESD and PHD were supported by NIH T32-HD007505. The research was funded by an award from Biogen to AL, by NIH P01-DK06041 and NCI P50-CA130810 project awards to LCS and Core support from the Michigan Gastrointestinal Research Center Grant NIH P30-DK34933 and the University of Michigan Cancer Center Support Grant NCI P30-CA6592.

COMPETING INTERESTS

CWS is an employee of Genentech Inc. and owns shares of Roche. All other authors state that they have no conflicts of interest.

Supplementary Table 2-1. Antibodies and lectins used for histological analysis

Antibody	Species/Clonality	Source	Dilution	Antigen Retrieval*
Chloride channel accessory 1 (CLCA1)	Rabbit/Monoclonal	Abcam (ab180851)	1:500	Antigen Unmasking Solution
Chromogranin A (CHGA)	Rabbit/Polyclonal	Abcam (15160)	1:200	
Cleaved-Caspase 3	Rabbit/Monoclonal	Cell Signaling (9661)	1:50	Tris-EDTA
Doublecortin-like kinase 1 (DCLK1)	Rabbit/Polyclonal	Abcam (ab37994)	1:50	
E-cadherin	Rat/Monoclonal	Invitrogen (13-1900)	1:1000	Tris-EDTA
Gastrin	Rabbit/Polyclonal	DAKO (A0568)	1:1000	Tris-EDTA
GFP	Rabbit/Polyclonal	Invitrogen (A21311)	1:200	
GFP	Chicken/Polyclonal	Abcam (ab13970)	1:200	Tris-EDTA
Griffonia simplicifolia II (GSII)	Lectin	Molecular Probes (L-21415)	1:1000	Antigen Unmasking Solution
Intrinsic Factor (GIF)	Rabbit/Polyclonal	Dr. D Alpers, Washington University	1:1000	Antigen Unmasking Solution
Histidine decarboxylase (HDC)	Rabbit/Polyclonal	Cappel (11670)	1:1000	Tris-EDTA
Ki67	Rabbit/Monoclonal	Thermo Scientific (RM-9106)	1:200	Tris-EDTA
Mucin 2 (MUC2)	Rabbit/Polyclonal	Santa Cruz (sc-15334)	1:200	Tris-EDTA
Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1)	Rat IgG	BD Biosciences (557355)	1:1000	
RFP	Rabbit/Polyclonal	Rockland Immunochemicals (600-401-379)	1:200	Trilogy
α -smooth muscle actin (α SMA)	Mouse/Monoclonal	Sigma-Aldrich (C6198)	1:500	

*Antigen retrieval was performed on paraffin sections only with Antigen Unmasking Solution (Vector), Trilogy (Cell Marque), or Tris-EDTA (10mM Trizma Base, 1mM EDTA, 0.05% Tween-20, pH 9.0). Staining in cryo sections did not use antigen retrieval.

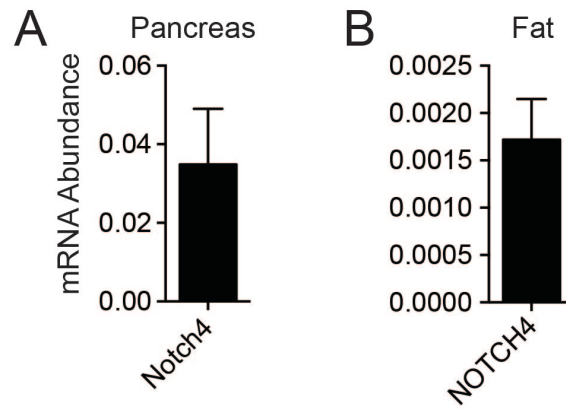
Supplementary Table 2-2. Oligonucleotide sequences used for qRT-PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Product Size
<i>Gapdh</i>	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGGTCTGGGATGG	350
<i>Notch1</i>	AGCAAGAAGAAGCGGAGAGAGC	TGTCGTCCATCAGAGCACCATC	91
<i>Notch2</i>	TGTGGAAGGAATGGTGGCAGAG	CTCGGGCAGCAAGAAACAAAGG	194
<i>Notch3</i>	TGATCGGCTCTGTGGTATGC	GGTGCTGTGTTCTCGCTTTTCG	290
<i>Notch4</i>	ATTCCTCTTCATGGGAAAGACA	ACTCCCATCACTACCACAAACC	91
<i>Gif</i>	CTTGGCCCTGACCTGTATGT	TAGGTTGCTCAGGTGTCACG	191
<i>Tff2</i>	TGCTTTGATCTTGGATGCTG	GGAAAAGCAGCAGTTTTCGAC	174
<i>Gast</i>	GGACCAGGGACCAATGAGG	CCAAAGTCCATCCATCCGTAGG	173
<i>Chga</i>	AAGAAGAGGAGGAGGAAGAGG	TCCATCCACTGCCTGAGAG	149
<i>Clca1</i>	CCAAAGGAAAACCCCAAGCAGTG	CCCGTTACTCTGTGCGATTACACAC	121
<i>Tff3</i>	TTGCTGGGTCCT CTGGGATA	GCCGGCACCATACATTGG	67
<i>Muc2</i>	AGAACGATGCCT ACACCAAG	CATTGAAGTCCCCGCAGAG	132
<i>Reg3γ</i>	AGGTGAAGTTGCCAAGAAAGATGC	ATCATGGAGGACAGGAAGGAAGC	201
<i>Hdc</i>	CGACTAAGAAGCCCTGTTGAC	TCACGGTATTCACGGTATTCAC	109
<i>Mmp7</i>	CAGACTTACCTCGGATCGTAGTGG	GTTCACCTCCTGCGTCCTCACC	269
<i>NOTCH1*</i>	GACAGCCTCAACGGGTACAA	CACACGTAGCCACTGGTCAT	137
<i>NOTCH2*</i>	CAACCGCCAGTGTGTTCAAG	GAGCCATGCTTACGCTTTTCG	240
<i>NOTCH3*</i>	TCTTGCTGCTGGTCATTCTC	TGCCTCATCCTCTTCAGTTG	485
<i>NOTCH4*</i>	TGAGGTGAATCCAGACAAC	ATACAGTCATCCAGGTTCTC	261
<i>ACTB*</i>	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGATAGCAAC	211

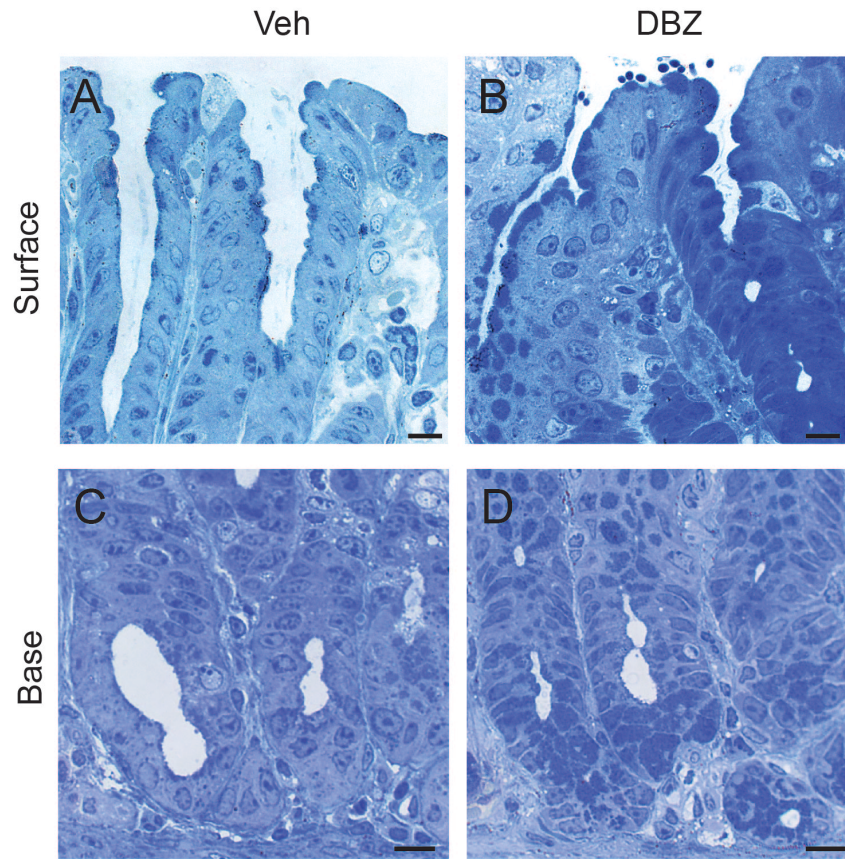
*Sequences designed to amplify the human gene; all other primers amplify the mouse gene

Supplementary Table 2-3. List of patient information for human tissues

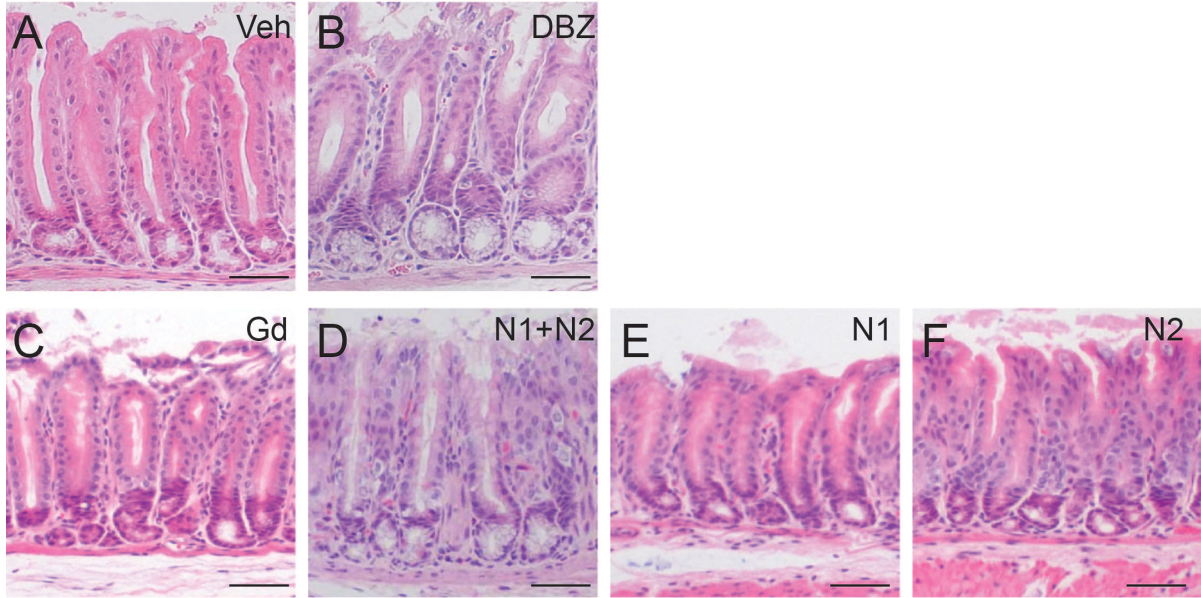
Patient	Age	Gender
H7	67	M
H29	59	M
H36	56	M
H41	80+	F
H46	68	M



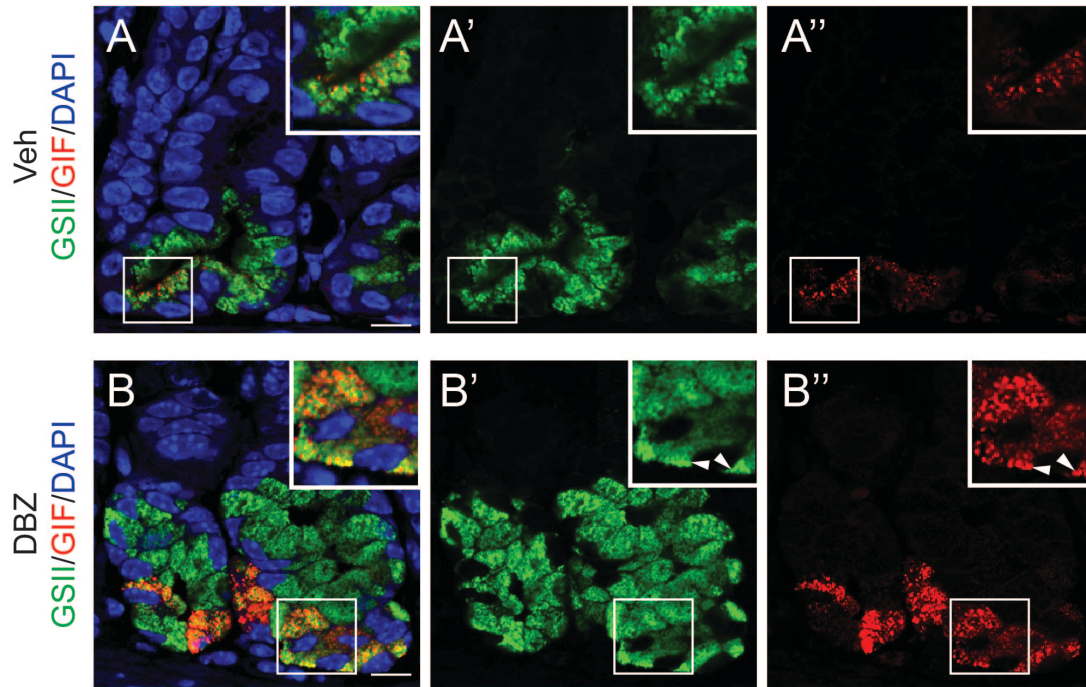
Supplementary Figure 2-1. Positive control for Notch4 qRT-PCR assay. mRNA abundance measured in (A) pancreatic tissue (mouse) or (B) fat tissue (human) (mean \pm SEM of technical replicates, n=1).



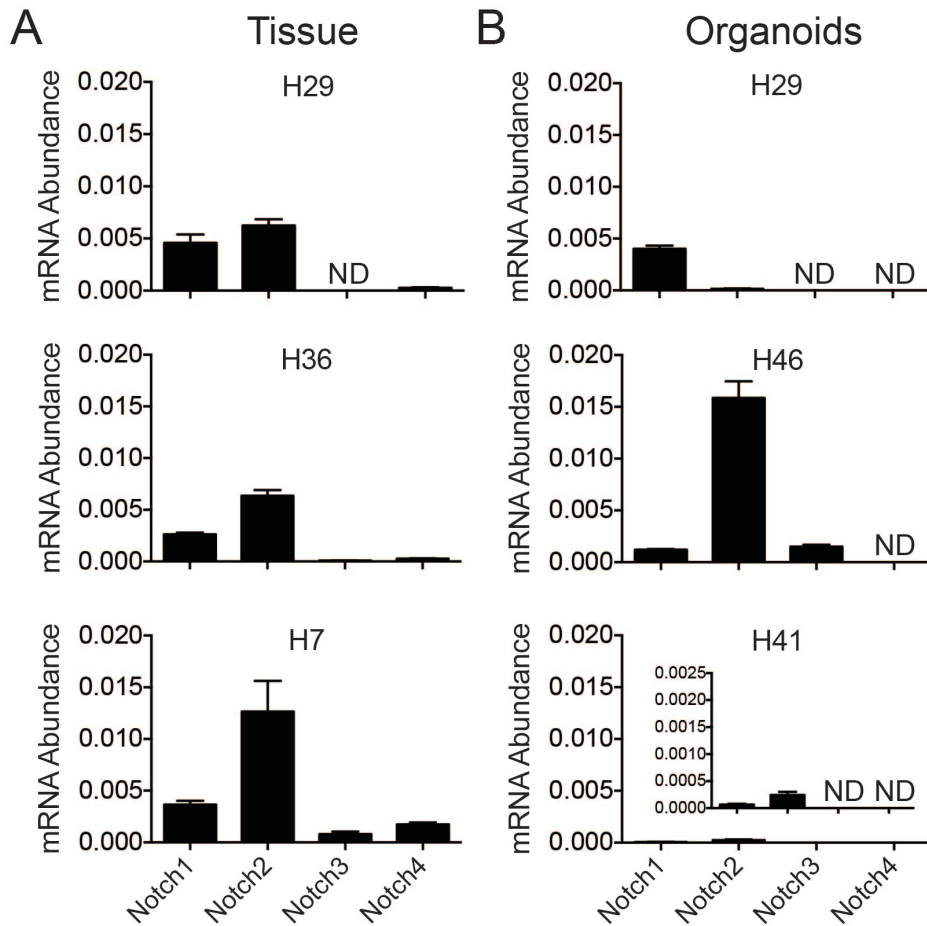
Supplementary Figure 2-2. Altered cellular morphology with Notch inhibition. Semi-thin sections of antral gland base and surface regions from (A,C) vehicle or (B,D) DBZ-treated mice stained with toluidine blue. Scale = 10 μ m.



Supplementary Figure 2-3. Antral gland histology after Notch inhibition. Paraffin sections stained with haematoxylin and eosin (H&E) from (A) vehicle, (B) DBZ, (C) α Gd, (D) α N1+ α N2, (E) α N1, or (F) α N2-treated mice. Scale bar: 50 μ m.

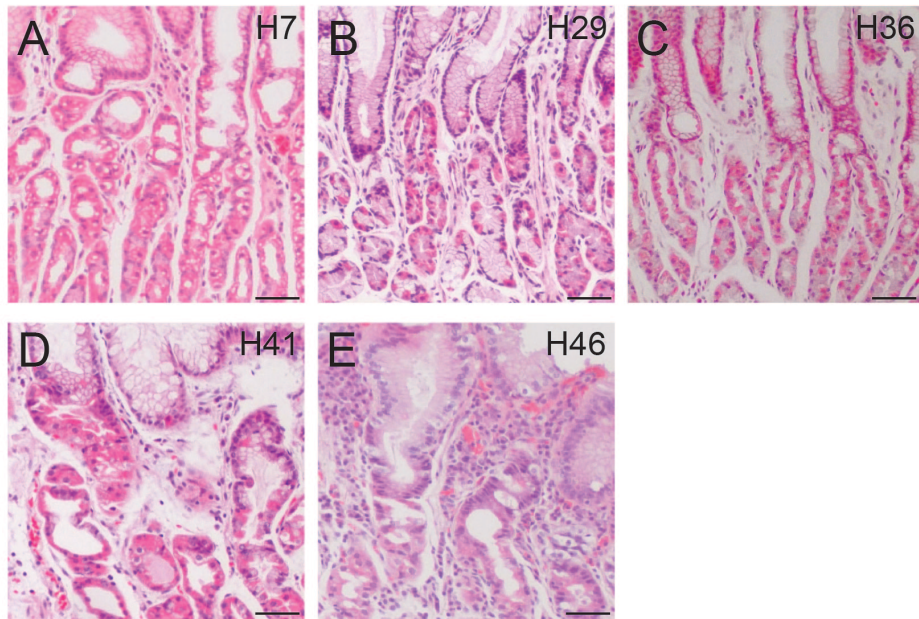


Supplementary Figure 2-4. Notch inhibition leads to increased GSII/GIF co-staining at the antral gland base. Confocal microscopy of paraffin tissue sections co-stained for GSII and GIF in (A) vehicle and (B) DBZ-treated mice. Occasional co-stained cells were detected in vehicle treated mice at the gland base. There was a marked increase in co-stained cells after Notch inhibition. Separate channels for GSII (A', B') and GIF (A'', B'') are shown. Although the majority of granules appear to be specific for GSII or GIF, there were also some co-stained granules (arrowheads). Scale bar: 10 μ m.



Supplementary Figure 2-5. Notch receptor expression in human gastric tissue.

Notch receptor mRNA abundance was measured in (A) full-thickness antral tissue samples and (B) established organoids. Patient samples are listed in Supplementary 2-3. Shown are results of qRT-PCR analysis in each patient tissue sample or organoid line as marked (mean \pm SEM of technical replicates). ND = Not detected.



Supplementary Figure 2-6. Histology of human gastric tissue samples used in this manuscript confirms antral tissue identity. Paraffin sections from individual human samples were stained with haematoxylin and eosin (H&E). Scale = 50 μ m.

REFERENCES

1. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R, Clevers H. Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. *Cell Stem Cell*. 2010;6(1):25-36. doi:10.1016/j.stem.2009.11.013.
2. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*. 2007;449(7165):1003-1007. doi:10.1038/nature06196.
3. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature*. 2005;435(June):959-963. doi:10.1038/nature03659.
4. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S. Notch signals control the fate of immature progenitor cells in the intestine. *Nature*. 2005;435(7044):964-968. doi:10.1038/nature03589.
5. Riccio O, van Gijn ME, Bezdek AC, Pellegrinet L, van Es JH, Zimmer-Strobl U, Strobl LJ, Honjo T, Clevers H, Radtke F. Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep*. 2008;9(4):377-383. doi:10.1038/embor.2008.7.
6. VanDussen KL, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kolterud Å, Grosse AS, Gumucio DL, Ernst S a, Tsai Y-H, Dempsey PJ, Samuelson LC. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. *Development*. 2012;139(3):488-497. doi:10.1242/dev.070763.
7. Carulli AJ, Keeley TM, Demitrack ES, Chung J, Maillard I, Samuelson LC. Notch receptor regulation of intestinal stem cell homeostasis and crypt regeneration. *Dev Biol*. 2015;402(1):98-108. doi:10.1016/j.ydbio.2015.03.012.
8. Kim T-H, Shivdasani R a. Notch signaling in stomach epithelial stem cell homeostasis. *J Exp Med*. 2011;208(4):677-688. doi:10.1084/jem.20101737.

9. Demitrack ES, Gifford GB, Keeley TM, Carulli AJ, VanDussen KL, Thomas D, Giordano TJ, Liu Z, Kopan R, Samuelson LC. Notch signaling regulates gastric antral LGR5 stem cell function. *EMBO J*. 2015;34(20):2522-2536. doi:10.15252/embj.201490583.
10. Yeh TS, Wu CN, Hsu KW, Liao WJ, Yang MC, Li AFY, Wang AM, Kuo ML, Chi CW. The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res*. 2009;69(12):5039-5048. doi:10.1158/0008-5472.CAN-08-4021.
11. Sun Y, Gao X, Liu J, Kong QY, Wang XW, Chen XY, Wang Q, Cheng YF, Qu XX, Li H. Differential Notch1 and Notch2 expression and frequent activation of Notch signaling in gastric cancers. *Arch Pathol Lab Med*. 2011;135:451-458. doi:10.1043/2009-0665-OA.1.
12. Andersson ER, Sandberg R, Lendahl U. Notch signaling: simplicity in design, versatility in function. *Development*. 2011;138(17):3593-3612. doi:10.1242/dev.063610.
13. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999;284(April):770-776. doi:10.1126/science.284.5415.770.
14. Tran IT, Sandy AR, Carulli AJ, Ebens C, Chung J, Shan GT, Radojicic V, Friedman A, Gridley T, Shelton A, Reddy P, Samuelson LC, Yan M, Siebel CW, Maillard I. Blockade of individual notch ligands and receptors controls graft-versus-host disease. *J Clin Invest*. 2013;123(4):1590-1604. doi:10.1172/JCI65477.
15. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, Finkle D, Venook R, Wu X, Ridgway J, Schahin-Reed D, Dow GJ, Shelton A, Stawicki S, Watts RJ, Zhang J, Choy R, Howard P, Kadyk L, Yan M, Zha J, Callahan C a, Hymowitz SG, Siebel CW. Therapeutic antibody targeting of individual Notch receptors. *Nature*. 2010;464(7291):1052-1057. doi:10.1038/nature08878.
16. Fre S, Hannezo E, Sale S, Huyghe M, Lafkas D, Kissel H, Louvi A, Greve J, Louvard D, Artavanis-Tsakonas S. Notch lineages and activity in intestinal stem cells determined by a new set of knock-in mice. *PLoS One*. 2011;6(10):e25785. doi:10.1371/journal.pone.0025785.

17. Nowotschin S, Xenopoulos P, Schrode N, Hadjantonakis A-K. A bright single-cell resolution live imaging reporter of Notch signaling in the mouse. *BMC Dev Biol.* 2013;13(1):15. doi:10.1186/1471-213X-13-15.
18. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci.* 2010;13(1):133-140. doi:10.1038/nn.2467.
19. Lopez-Diaz L, Hinkle KL, Jain RN, Zavros Y, Brunkan CS, Keeley T, Eaton K a, Merchant JL, Chew CS, Samuelson LC. Parietal cell hyperstimulation and autoimmune gastritis in cholera toxin transgenic mice. *Am J Physiol Gastrointest Liver Physiol.* 2006;290(5):G970-G979. doi:10.1152/ajpgi.00461.2005.
20. Keeley TM, Samuelson LC. Cytodifferentiation of the postnatal mouse stomach in normal and Huntingtin-interacting protein 1-related-deficient mice. *Am J Physiol Gastrointest Liver Physiol.* 2010;299(6):G1241-G1251. doi:10.1152/ajpgi.00239.2010.
21. Miyoshi H, Stappenbeck TS. In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. *Nat Protoc.* 2013;8(12):2471-2482. doi:10.1038/nprot.2013.153.
22. VanDussen KL, Marinshaw JM, Shaikh N, Miyoshi H, Moon C, Tarr PI, Ciorba MA, Stappenbeck TS. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut.* 2015;64(6):911-920. doi:10.1136/gutjnl-2013-306651.
23. Chen X, Leung S, Yuen S, Chu K-M, Ji J, Li R, Chan A, Law S, Troyanskaya O, Wong J, So S, Boystein D, Brown P. Variation in Gene Expression Patterns in Human Gastric Cancers. *Mol Biol Cell.* 2003;14(August):3208-3215. doi:10.1091/mbc.E02.
24. Du X, Cheng Z, Wang Y-H, Guo Z-H, Zhang S-Q, Hu J-K, Zhou Z-G. Role of Notch signaling pathway in gastric cancer: A meta-analysis of the literature. *World J Gastroenterol.* 2014;20(27):9191-9199. doi:10.3748/wjg.v20.i27.9191.
25. Schmidt PH, Lee JR, Joshi V, Playford RJ, Poulsom R, Wright NA, Goldenring JR. Identification of a metaplastic cell lineage associated with human gastric adenocarcinoma. *Lab Invest.* 1999;79(6):639-646. <http://www.ncbi.nlm.nih.gov/pubmed/10378506>. Accessed August 21, 2015.

26. Halldórsdóttir AM, Sigurdardóttir M, Jónasson JG, Oddsdóttir M, Magnússon J, Lee JR, Goldenring JR. Spasmolytic polypeptide-expressing metaplasia (SPEM) associated with gastric cancer in Iceland. *Dig Dis Sci*. 2003;48(3):431-441. <http://www.ncbi.nlm.nih.gov/pubmed/12757153>. Accessed August 21, 2015.
27. Nozaki K, Ogawa M, Williams JA, LaFleur BJ, Ng V, Drapkin RI, Mills JC, Konieczny SF, Nomura S, Goldenring JR. A molecular signature of gastric metaplasia arising in response to acute parietal cell loss. *Gastroenterology*. 2008;134(2):511-522. doi:10.1053/j.gastro.2007.11.058.
28. Weis VG, Sousa JF, LaFleur BJ, Nam KT, Weis JA, Finke PE, Ameen NA, Fox JG, Goldenring JR. Heterogeneity in mouse spasmolytic polypeptide-expressing metaplasia lineages identifies markers of metaplastic progression. *Gut*. 2012;62(9):1270-1279. doi:10.1136/gutjnl-2012-302401.
29. Bartfeld S, Bayram T, van de Wetering M, Huch M, Begthel H, Kujala P, Vries R, Peters PJ, Clevers H. In Vitro Expansion of Human Gastric Epithelial Stem Cells and Their Responses to Bacterial Infection. *Gastroenterology*. 2015;148(1):126-136.e6. doi:10.1053/j.gastro.2014.09.042.
30. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. 2009;459(7244):262-265. doi:10.1038/nature07935.
31. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol*. 2015;12(8):445-464. doi:10.1038/nrclinonc.2015.61.