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## Notch1 and Notch2 receptors regulate mouse and human gastric antral epithelial cell homeostasis

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### Abstract

**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

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**Competing interests** CWS is an employee of Genentech and owns shares of Roche.

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of all gastric antral lineages, with remodelling of cells to express secretory products normally associated with other regions of the GI tract, including intestine. Analysis of mouse and human organoids showed that Notch signalling through Notch1 and Notch2 is intrinsic to the epithelium and required for organoid growth.

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

## 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.<sup>12</sup> Antral LGR5 stem cells give rise to all antral lineages, including surface mucous cells, endocrine cells and deep mucous cells. The signalling pathways regulating gastric stem cell proliferation and differentiation are currently poorly understood.

### Significance of this study

#### What is already known on this subject?

- Notch signalling controls mouse gastric epithelial cell homeostasis.
- Mouse antral LGR5 stem cell function is regulated by Notch.
- Constitutive Notch activation in mice leads to gastric tumours.
- Expression of Notch components is increased in some human gastric cancers.

#### What are the new findings?

- Notch1 and Notch2 are the primary receptors mediating Notch effects in the mouse antrum.
- Antral LGR5 stem cells are regulated by Notch1 and Notch2.
- Notch inhibition induces antral cell remodelling to express corpus and intestinal markers.
- Human gastric antral organoid growth is regulated by Notch1 and Notch2.

#### How might it impact on clinical practice in the foreseeable future?

- Activation of the Notch signalling pathway may contribute to the pathogenesis of human gastric proliferative diseases.
- Targeting the Notch signalling pathway to treat human disease might disturb gastric epithelial cell homeostasis. Thus GI side effects need to be taken into account to evaluate the effectiveness of therapeutic interventions that target Notch.

Notch signalling is well described to maintain intestinal stem cells,<sup>3–7</sup> and recent studies suggest that gastric stem cells are similarly regulated by Notch.<sup>89</sup> 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) induced stem cell proliferation, gland fission and ultimately hyperproliferative polyps.<sup>89</sup> Furthermore, increased expression of Notch signalling components has been associated with gastric cancer, suggesting Notch pathway involvement.<sup>1011</sup>

Four Notch receptors (Notch1–4) exist in vertebrates that are single-pass transmembrane proteins.<sup>12</sup> Receptor signalling involves proteolytic receptor cleavage to release the intracellular signalling component NICD, which activates target gene transcription, such as those in the *Hes* and *Hey* families.<sup>13</sup> Notch1 and Notch2 are the primary receptors involved in intestinal stem cell homeostasis, with Notch1 having a predominant function.<sup>571415</sup> Global pharmacological Notch inhibition leads to intestinal toxicity,<sup>3</sup> but inhibition of Notch1 alone revealed a partial Notch-inhibition phenotype while avoiding major toxicity.<sup>71415</sup>

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 Notch1 and Notch2 function to regulate growth of antral organoid cultures generated from human and mouse tissue.

## METHODS

### Mice

Mice of both sexes aged 2–3 months were used. *Lgr5-EGFP-IRES-CreERT2* (*Lgr5-green fluorescent protein (GFP)*),<sup>2</sup> (Jackson Labs #008875), *Notch1-CreERT2<sup>SAT</sup>* (*NI*Cre) and *Notch2-CreERT2<sup>SAT</sup>* (*N2*Cre),<sup>16</sup> *CBF:H2B-Venus*,<sup>17</sup> (Jackson Labs #020942) and *ROSA26-CAG-LSL-tdTomato-WPRE* (*ROSA-Tom*),<sup>18</sup> (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-h light/dark cycle. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals. For lineage tracing Notch1-expressing or Notch2-expressing cells, *NI*Cre; *ROSA-Tom* and *N2*Cre; *ROSA-Tom* mice were treated with either one injection of tamoxifen (1 mg/20 g body weight) followed by a 3-day chase or five daily injections of tamoxifen followed by a 2-week chase.

### Notch pathway inhibition

For *in vivo* Notch inhibition, the  $\gamma$ -secretase inhibitor (GSI) dibenzazepine (DBZ, 30  $\mu$ mol/kg intraperitoneal, SYNCOM, Groningen, The Netherlands) or vehicle (0.1% Tween-80, 0.5% hydroxypropylmethylcellulose, 0.1% dimethyl sulfoxide (DMSO) in water)

was administered to *Lgr5-GFP* mice once per day for 5 days, with tissue collected the 6th day. Humanised IgG1 neutralising monoclonal antibodies specific for the Notch1-negative or Notch2-negative regulatory region ( $\alpha$ N1 or  $\alpha$ N2), or an irrelevant control IgG1 antibody interacting with herpes simplex virus gD protein ( $\alpha$ Gd) were described previously.<sup>15</sup> Antibodies were injected intraperitoneally at 5 mg/kg on day 1 and day 4, with collection of stomach tissue on day 6, day 14 or day 28.

For in vitro treatment of mouse and human organoids, the GSI *N*-(*N*-(3,5-Difluorophenacetyl-L-alanyl)-(*S*)-phenylglycine t-butyl ester (DAPT, 1  $\mu$ M; EMD4Biosciences, Gibbstown, New Jersey, USA),  $\alpha$ Gd,  $\alpha$ N1 or  $\alpha$ N2 (10  $\mu$ 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, New York, USA) 1.5 h prior to tissue collection. Stomachs were processed for cryo and paraffin histology as described.<sup>9</sup> For analysis of proliferation, EdU incorporation was visualised with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Life Technologies, Carlsbad, California, USA). Tissue sections were incubated with primary antibodies (see online supplementary table S1) followed by appropriate secondary antibodies (1:400, Invitrogen) and mounted with ProLong Gold containing 4,6-diamidino-2-phenylindole dihydrochloride (4',6-diamidino-2-phenylindole dihydrochloride (DAPI); Invitrogen) as described.<sup>19</sup> To analyse proliferating LGR5 stem cells, sections were immunostained for GFP (chicken) and Ki67 as described.<sup>9</sup> Antral glands were isolated and immunostained as previously described.<sup>9</sup> 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 described.<sup>6</sup> 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 described.<sup>9</sup> 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 500 ng total RNA and quantitative RT-PCR was performed as described,<sup>20</sup> using primers listed in online supplementary table S2, normalised to *Gapdh* (mouse) or *ACTB* (human).

### Gastric organoid culture

Mouse gastric organoid culture was carried out as described.<sup>9,21</sup> In brief, antral glands were resuspended in Matrigel (Corning, Tewksbury, Massachusetts, USA) and overlaid with culture media (50% L cell line expressing Wnt3a, R-spondin3, Noggin (L-WRN) conditioned media, Advanced Dulbecco's modified eagle's medium (DMEM)/F12, 10% fetal bovine serum (FBS), 1X Pen-Strep, 2 mM L-Glutamine), with the addition of Y-27632 (10  $\mu$ M, Tocris, Bristol, UK) only upon initial plating. L-WRN conditioned media containing Wnt3a, R-spondin3 and Noggin was generated as described.<sup>21</sup> Human antral

tissue (surgical resection or Gift of Life) was obtained under Institutional Review Board approved protocols and organoids were established as described,<sup>22</sup> with modifications. Tissue was incubated with 10 mM dithiothreitol (Invitrogen) in Dulbecco's phosphate-buffered saline (DPBS) with Pen-Strep and Gentamycin for 15 min at room temperature, followed by incubation in 12 mM EDTA in DPBS with Pen-Strep and Gentamycin for 1 h 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, the 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 three independent established organoid lines that had been passaged at least three times before analysis.

### Morphometrics

Morphometric analysis used ImageJ software (1.46r, Wayne Rasband, National Institutes of Health (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 normalised 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 with control (vehicle or αGd) organoids.

### Statistics

GraphPad Prism software was used for statistical analysis. Quantitative data are presented as mean±SEM and analysed using Student's t test (comparing DBZ/DAPT to vehicle) or one-way analysis of variance (ANOVA) with Dunnett's or Tukey's post hoc test (comparing αN1, αN2, αN1+αN2 and αGd); p<0.05 was considered statistically significant (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001). qRT-PCR data are expressed as mRNA fold-change versus control (vehicle or αGd).

## RESULTS

### Notch signalling in antral stem cells

We used the *CBF:H2B-Venus* reporter mouse to identify cells undergoing Notch signalling in the gastric antrum. This transgenic mouse expresses a nuclear fluorescent protein in response to Notch receptor activation.<sup>17</sup> Isolated antral glands showed scattered Venus-positive cells that were primarily located in the gland base (figure 1A). Costaining showed Venus expression in differentiated cell types, including chromogranin A-expressing enteroendocrine cells (figure 1B) and doublecortin like kinase 1-expressing tuft cells (figure 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 signalling in antral LGR5 stem cells (figure 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 (figure 1E, see online supplementary figure S1). 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 (figure 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.

In addition to epithelial cell labelling there was extensive non-epithelial Notch lineage marking with *N1Cre;ROSA-Tom* and *N2Cre;ROSA-Tom* (figure 1F, arrowheads). Histological analysis of tissue sections from the *CBF:H2B-Venus* reporter mouse demonstrated that the majority of Venus-positive stromal cells are Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1)-expressing endothelial cells and  $\alpha$ -smooth muscle actin-expressing smooth muscle cells (figure 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 GSI DBZ (a pan-Notch inhibitor). Analysis of EdU incorporation showed that proliferation was reduced with combined  $\alpha$ N1+ $\alpha$ N2 treatment to a similar extent as DBZ (figure 2A–H). Treatment with  $\alpha$ N1 or  $\alpha$ N2 alone significantly reduced the number of proliferating cells, but to a lesser extent (figure 2D–H). Analysis at 2 weeks and 4 weeks after  $\alpha$ N1 or  $\alpha$ N2 treatment demonstrated normal proliferation after the antibody treatment was discontinued (figure 2H). However, morbidity prevented analysis of the  $\alpha$ N1+ $\alpha$ N2 group at these later time points.

To directly investigate LGR5 stem cells, we treated *Lgr5-GFP* mice with  $\alpha$ N1 and/or  $\alpha$ N2 and analysed stem cell proliferation via coimmunostaining for GFP and Ki67 (figure 2I, arrow). Notch1 and/or Notch2 inhibition reduced LGR5 stem cell proliferation, with  $\alpha$ N1+ $\alpha$ N2 having the most substantial effect (figure 2J), which was similar to our previous analysis of pan-Notch inhibition with DBZ.<sup>9</sup> 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 analysed 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 in the gland base (figure 3). As with proliferation,  $\alpha$ N1+ $\alpha$ N2 treatment was comparable to DBZ, with more modest effects observed after treatment with antibodies targeting individual receptors (figure 3C, D).

## Notch regulates antral organoid growth

We established organoids to test whether the Notch effects are intrinsic to epithelial cells. Organoids established from the *CBF: H2B-Venus* reporter mouse showed extensive Venus labelling, demonstrating active Notch signalling in organoid culture (figure 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 (figure 4B). To investigate Notch function we treated organoids with the pan-Notch inhibitor DAPT and observed reduced overall growth (figure 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 (figure 4F–J). These findings demonstrate that intrinsic Notch signalling is required for antral organoid growth.

## Notch1 and Notch2 regulate antral epithelial cell differentiation

We previously showed that Notch inhibition was associated with increased antral cell differentiation.<sup>9</sup> Thus, we analysed 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 (figure 5). Similar effects were observed after treatment with DBZ (figure 5A–E) and  $\alpha$ N1 +  $\alpha$ N2, but not with  $\alpha$ N1 or  $\alpha$ N2 treatment, suggesting that these two receptors are fully redundant for this function (figure 5F–L). In accordance with the *in vivo* findings, increased *Gast* mRNA was observed in organoids after DAPT or  $\alpha$ N1+ $\alpha$ N2 treatment (figure 5M, N).

Similarly, analysis of the surface mucous cell marker CLCA1 showed expansion of this cell population after DBZ (figure 6A–C) or combined  $\alpha$ N1+ $\alpha$ N2 treatment, but not after individual receptor blockade (figure 6D–H). Consistent with the marker expression data, ultrastructural analysis showed a marked expansion of surface mucous cells with increased numbers of granules in these cells after DBZ treatment (figure 6I, J; see online supplementary figure S2).

Analysis of H&E stained tissues suggested increased mucous cells at the gland base after Notch inhibition (see online supplementary figure S3). Accordingly, griffonia simplicifolia lectin II (GSII)-lectin staining showed an apparent expansion of this cell lineage after DBZ or  $\alpha$ N1+ $\alpha$ N2 treatment (figure 7A, B, E–H), which was confirmed by increased trefoil factor 2 (*Tff2*) mRNA abundance (figure 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 (figure 7D, J). High-powered confocal analysis showed GSII and GIF colabelled cells at the gland base, primarily localised in separate granules (see online supplementary figure S4). Ultrastructural analysis showed a marked increase in secretory granule number and granule size in cells at the gland base after Notch inhibition (figure 7M, N and see online supplementary figure S2). Consistent with the *in vivo* data, increased *Gif* expression was also observed in DAPT or  $\alpha$ N1+ $\alpha$ N2 treated antral organoids (figure 7K, L).

To better understand the cellular remodelling 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 enterochromaffin-like (ECL) cell marker histidine decarboxylase (figure 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 $\gamma$* ) in the DBZ-treated antrum (figure 8D–J). Immunostaining revealed scattered Muc2-positive cells in the distal antrum that were localised to the mid-region of the gland. Similar to the corpus markers, the increase in intestinal markers was not associated with a general intestinalisation as the DBZ-treated antrum did not increase expression of the intestinal markers *Cdx2*, *Vill*, *Cck* or *Lyz1* (data not shown) and continued to express gastric specific markers (*Tff2* and *Gif*).

Together these findings suggest that global Notch inhibition with DBZ or combined  $\alpha$ N1+ $\alpha$ N2 treatment results in a generalised increase in cellular differentiation, with some cellular remodelling to express markers of other regions of the GI tract. Dual receptor blockade is required for the differentiation effect, suggesting that Notch1 and Notch2 function together during differentiation.

### Notch is necessary for growth of human gastric organoids

We took advantage of the design of the inhibitory antibodies to target 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 (figure 9A, B and see online supplementary figures S5 and S6, table S3).

We confirmed that intrinsic Notch signalling was required for growth of human organoids, with reduced organoid size observed after addition of DAPT to the culture media (figure 9C–E). Treatment with  $\alpha$ N1+ $\alpha$ N2,  $\alpha$ N1 or  $\alpha$ 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.

## DISCUSSION

Here we report that Notch signalling regulates gastric antral epithelial cell homeostasis through the Notch1 and Notch2 receptors. Inhibition of Notch1 and Notch2 signalling mimicked effects on proliferation and differentiation observed with global Notch inhibition with DBZ treatment. Notch signalling in antral LGR5 stem cells was shown by Notch reporter expression and lineage tracing studies. We also showed that Notch signalling is crucial for proliferation of gastric LGR5 stem cells, with Notch1 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 *NI*Cre; *ROSA-Tom* mice but only short-term epithelial cell marking in *N2*Cre; *ROSA-Tom* mice. In addition  $\alpha$ N1 was more effective than  $\alpha$ 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 signalling 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,<sup>101123</sup> with NOTCH1 being associated with diffuse type and JAGGED1 being overexpressed in diffuse and poorly differentiated type cancers.<sup>24</sup> Consistent with a potential role for Notch in promoting gastric tumorigenesis, genetic mouse models of Notch overexpression in the stomach induced hyper-plastic polyps.<sup>89</sup>

We further demonstrate that Notch1 and Notch2 regulate epithelial cell differentiation. Inhibition of Notch1 and Notch2 signalling 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.<sup>346</sup> Thus, while Notch signalling in the intestine promotes enterocyte differentiation, Notch signalling in the stomach appears to generally repress differentiation of all lineages.<sup>9</sup> 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 remodelling. 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 remodelling in the gland base was characterised 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 GI tissues. Further studies are needed to understand the role Notch plays in maintaining antral identity.

Cells that coexpress GIF and mucous cell markers, such as TFF2, have been previously described in immature and adult stomach. In mouse, coexpressing cells are observed throughout the immature newborn stomach, including the corpus and antral regions.<sup>20</sup> Moreover, in adult human and mouse corpus, a coexpressing metaplastic cell lineage termed spasmolytic polypeptide-expressing metaplasia (SPEM) has been observed.<sup>25</sup> SPEM is induced in response to loss of parietal cells and has been proposed to be a precursor to gastric cancer.<sup>26-28</sup> How the remodelled 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.<sup>1222930</sup> Either complete Notch blockade with GSI, or specific receptor targeting with  $\alpha$ N1 or  $\alpha$ 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 signalling in antral LGR5 stem cells. Furthermore these studies showed that Notch signalling is intrinsic to the gastric epithelium, with signalling 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  $\alpha$ N1+ $\alpha$ N2 treatment, similar to what we observed *in vivo*.

Our finding that Notch1 and Notch2 receptor signalling 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 significance.<sup>31</sup> The use of pan-Notch inhibitors such as GSIs for Notch-related pathologies has been of clinical relevance for years; however GI toxicity severely limits their use.<sup>3</sup> Thus more specific pathway targets are being explored. In particular, the development of humanised 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 inhibition.<sup>15</sup> 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.

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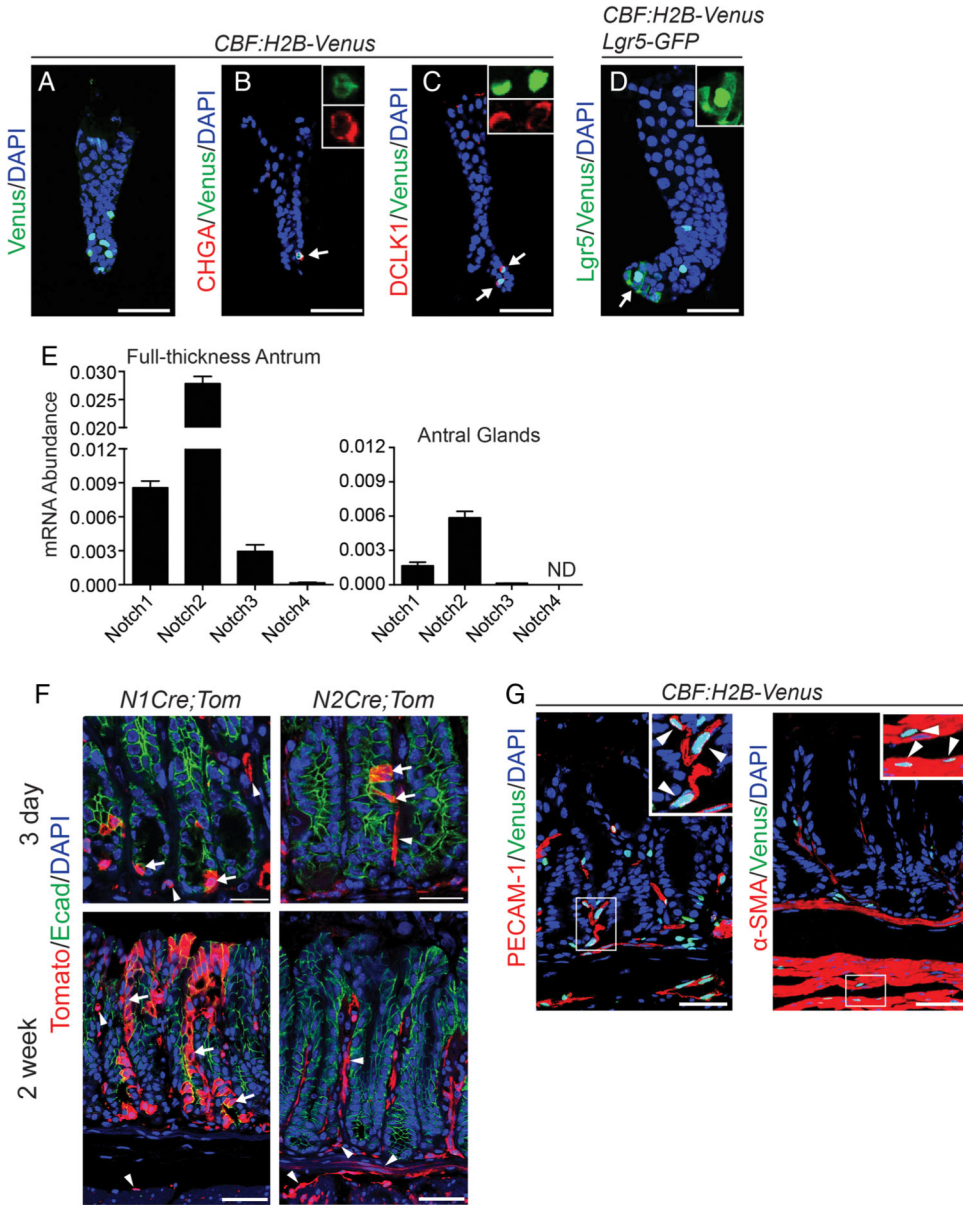
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**Figure 1.** Notch signalling in gastric antral cells. (A–D) Antral glands isolated from *CBF:H2B-Venus* mice (green, nuclear) costained for (B) chromogranin A (CHGA) (red), (C) doublecortin like kinase 1 (DCLK1) (red), or (D) *Lgr5*-GFP (green, cytoplasmic) 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±SEM; n=3–5 mice). ND, not detected. (F) Confocal imaging of paraffin sections from *N1-Cre;Tom* and *N2-Cre;Tom* mice costained for red fluorescent protein (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 costained

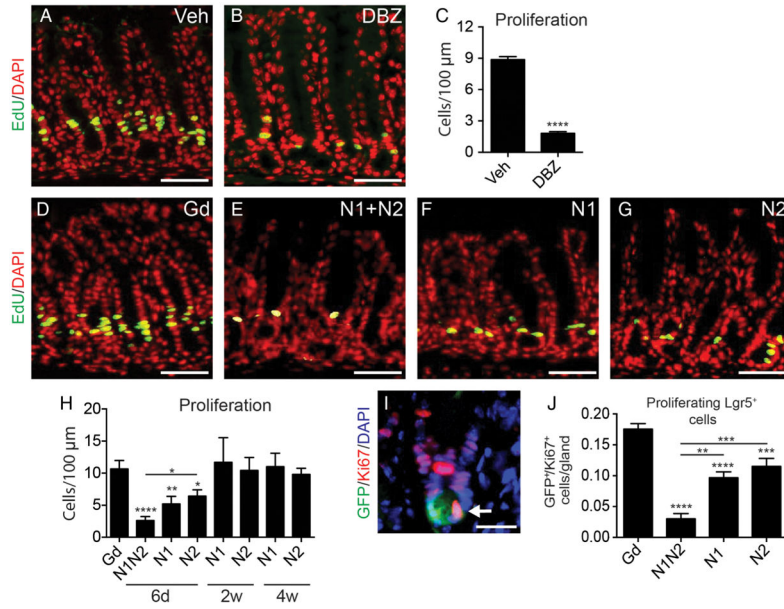
for the endothelial marker Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1) (left, red) or the smooth muscle cell marker  $\alpha$ -smooth muscle actin (SMA) (right, red). Costained cells in boxed regions are marked (arrowheads) in higher-powered insets. Scale bar: 50  $\mu$ m.

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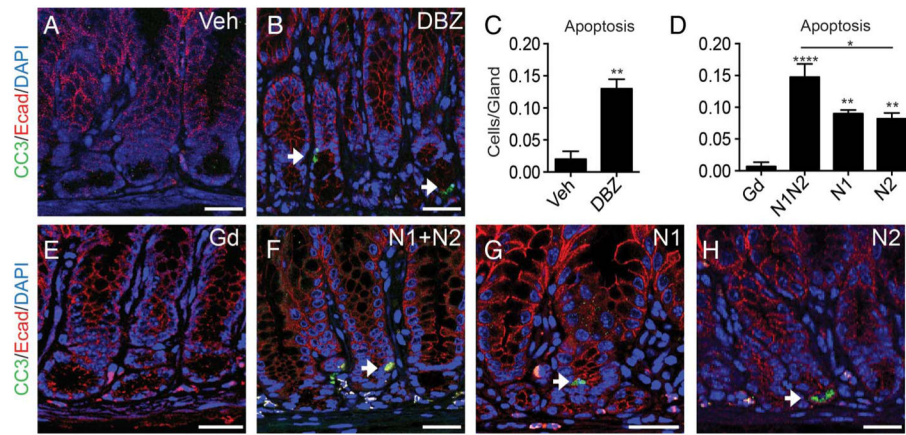
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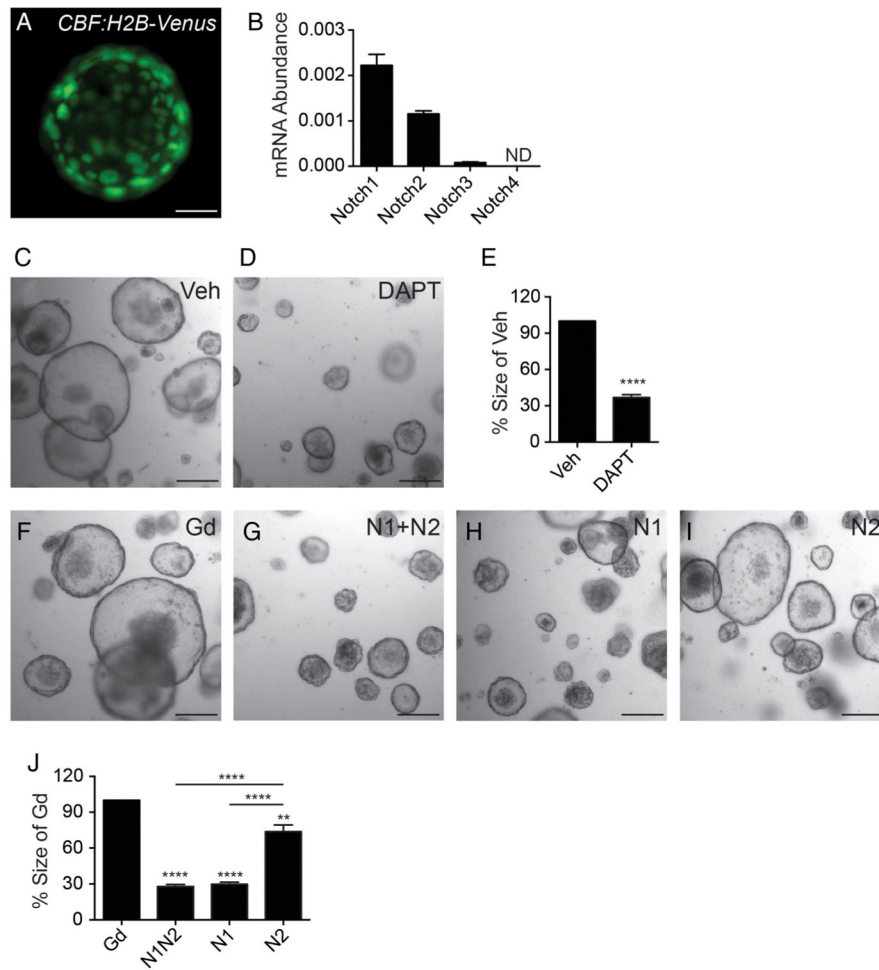
**Figure 2.** Notch1 and Notch2 regulate antral cell proliferation. (A, B and D–G) Proliferating cells were detected in (A) vehicle, (B) dibenzazepine (DBZ), (D) Gd antibody control (αGd), (E) αN1+αN2, (F) αN1 and (G) αN2 tissues by staining for EdU (green) with DAPI (red). (C and H) Morphometric quantification of EdU cells (mean±SEM; n=5–7 mice). (I and J) Proliferating LGR5 stem cells were visualised in treated *Lgr5-GFP* mice by GFP (green) and Ki67 (red) costaining (arrow) with DAPI (blue) and quantified by morphometrics (mean ±SEM; n=5–7 mice; 40–100 glands/mouse). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 versus vehicle using Student’s t test or versus αGd using one-way ANOVA. Scale bar: 50 μm (A, B and D–G) or 25 μm (I).



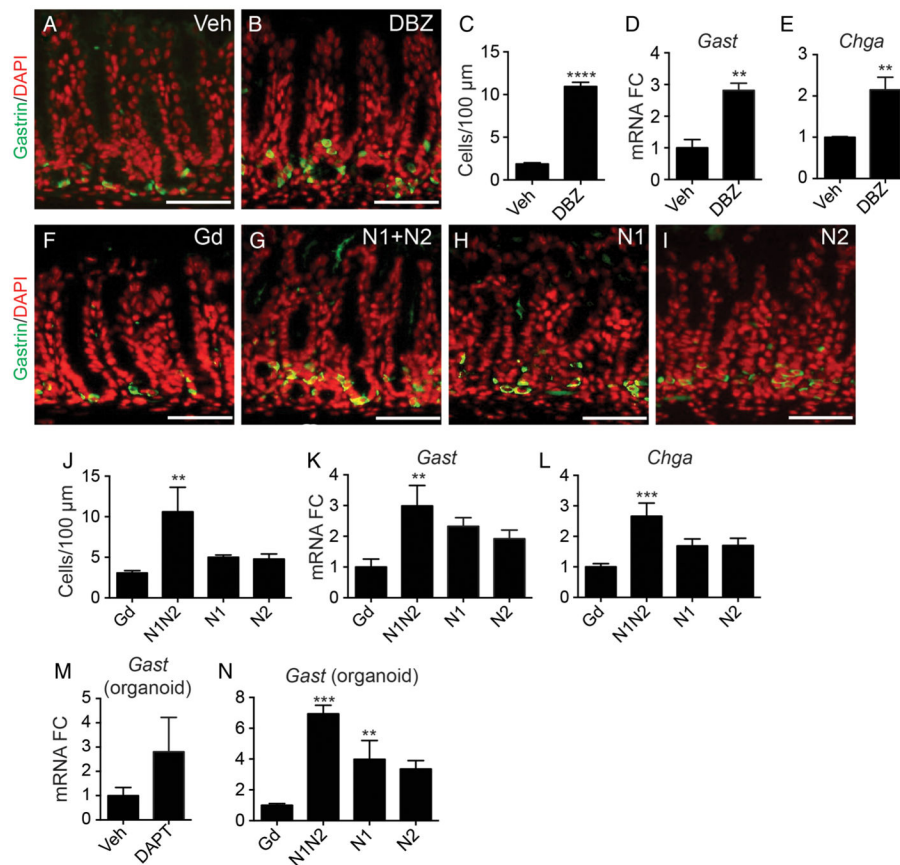
**Figure 3.**

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

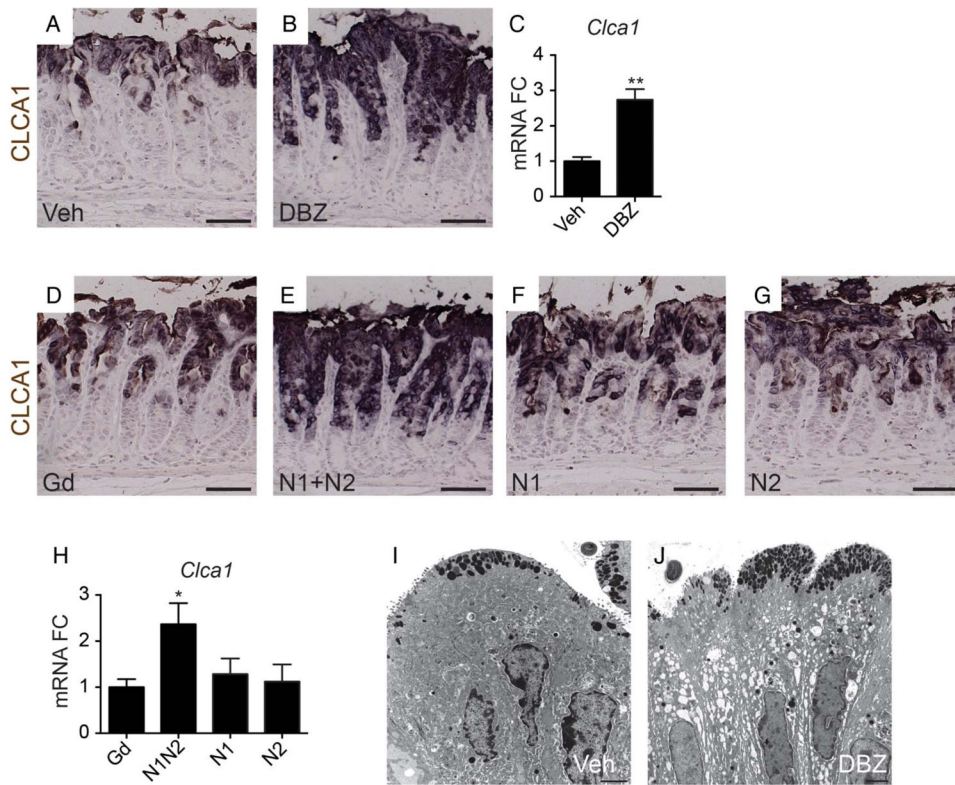




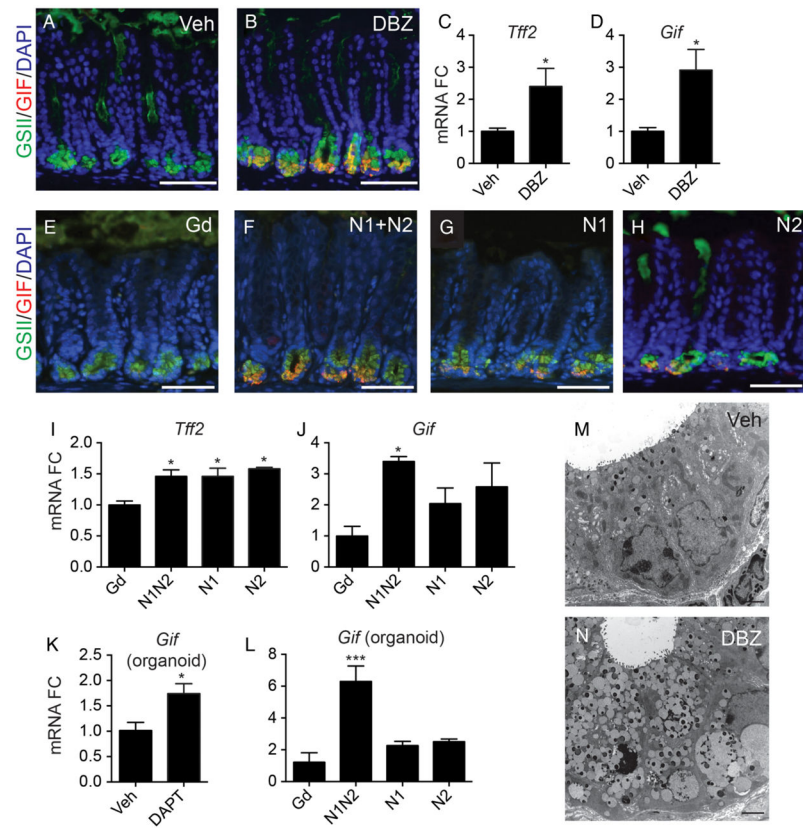
**Figure 4.** Notch1 and Notch2 regulate mouse antral organoid growth. (A) Cells undergoing Notch signalling (green) were observed in antral organoids established from the *CBF:H2B-Venus* Notch reporter mouse strain. (B) Gene expression of Notch receptors determined by qRT-PCR analysis of antral organoids (mean±SEM n=3 independent organoid lines). ND, not detected. Morphology (C, D and F–I) and quantitative measure of organoid size (E and J) following 5 days of treatment with (C) vehicle, (D) *N*-(*N*-(3,5-Difluorophenacetyl-L-alanyl))-(*S*)-phenylglycine t-butyl ester (DAPT), (F) αGd, (G) αN1+αN2, (H) αN1 or (I) αN2 (mean±SEM; n=3 lines). Scale bar: 50 μm (A) or 250 μm (C, D and F–I).

**Figure 5.**

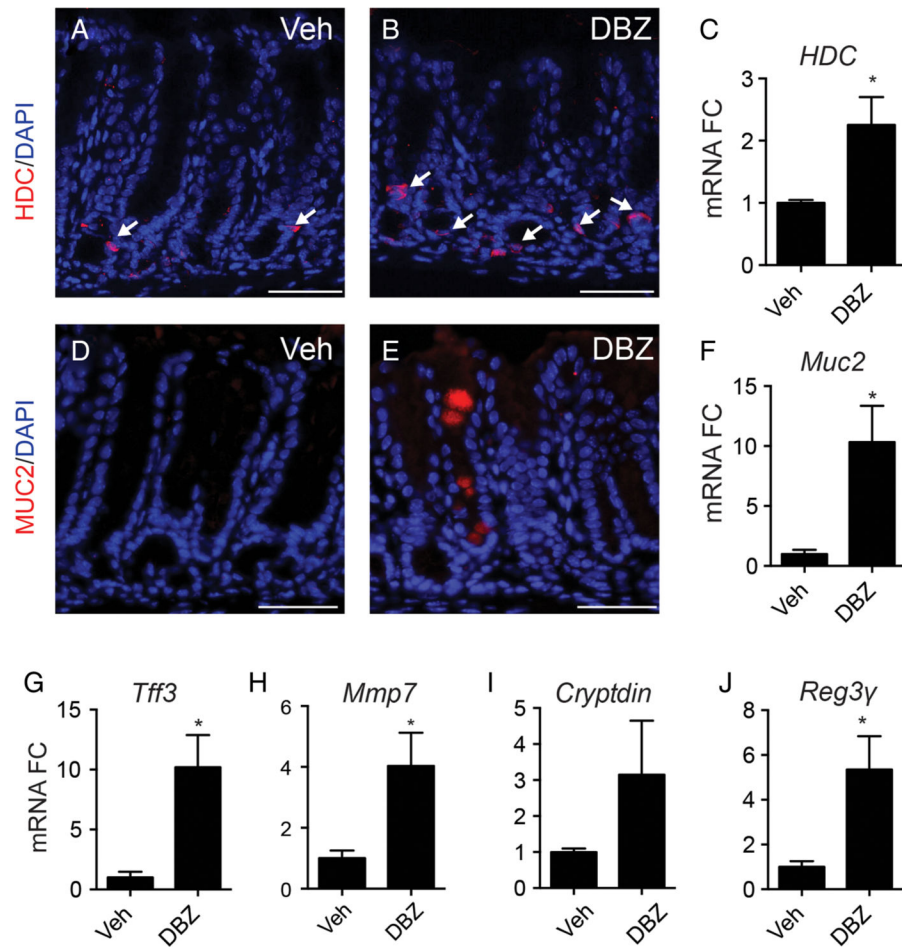
Notch1 and Notch2 regulate antral endocrine cell differentiation. (A, B and F–I) Gastrin immunostaining (green) of paraffin sections from mice treated with (A) vehicle, (B) dibenzazepine (DBZ), (F)  $\alpha$ Gd, (G)  $\alpha$ N1+ $\alpha$ N2, (H)  $\alpha$ N1, and (I)  $\alpha$ N2 with DAPI (red). (C and 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 and L) full-thickness tissue or (M and N) organoids (mean $\pm$ SEM; n=5–7 mice or 3 independent organoid lines). Scale bar: 50  $\mu$ m.



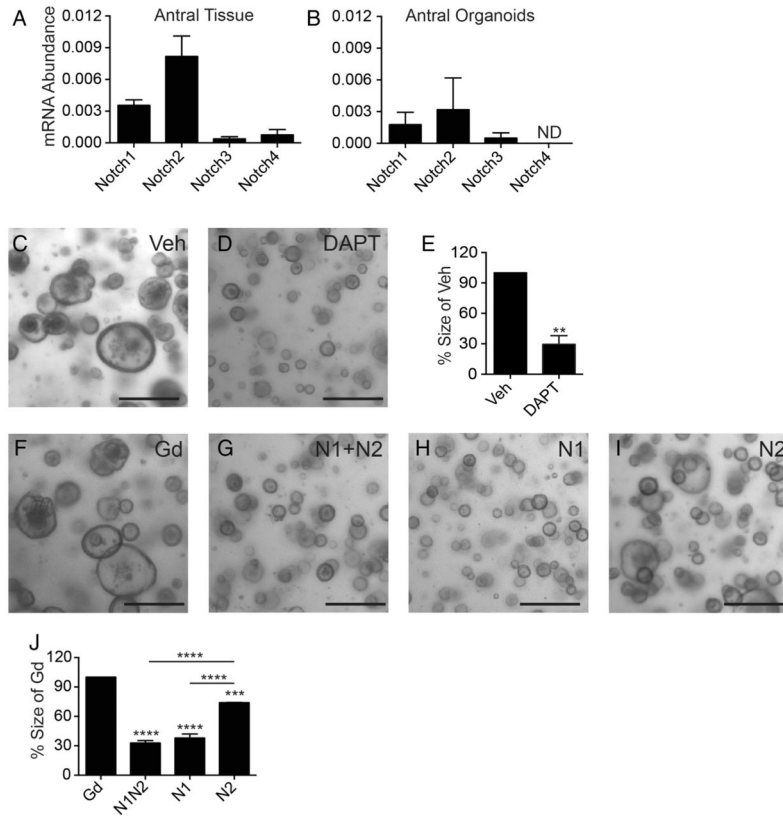
**Figure 6.** Notch1 and Notch2 regulate surface mucous cell differentiation. (A, B and D–G) Immunostaining for the surface mucous cell marker CLCA1 in (A) vehicle, (B) dibenzazepine (DBZ), (D) αGd, (E) αN1+αN2, (F) αN1 and (G) αN2-treated mice. (C and H) qRT-PCR analysis of *Clca1* expression (mean±SEM; n=5–7 mice). (I and J) Transmission electron microscope images of surface mucous cells from (I) vehicle or (J) DBZ-treated mice. Scale bar: 50 μm (A, B and D–G) or 2 μm (I and J).



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



**Figure 8.** Increased expression of gastric corpus and intestinal cell markers in dibenzazepine (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).

**Figure 9.**

Human antral organoid growth is regulated by Notch1 and Notch2. (A and B) Notch receptor mRNA abundance was measured by qRT-PCR in (A) full-thickness human tissue or (B) antral organoids (mean±SEM, n=3 patients or patient-derived organoid lines). See online supplementary figures S5 and S6, table S3 for detailed information on human tissues. Morphology (C, D and F–I) and quantitative measure of organoid size (E and J) following treatment with (C) vehicle, (D) *N*-(*N*-(3,5-Difluorophenacetyl-L-alanyl))-(*S*)-phenylglycine *t*-butyl ester (DAPT), (F) αGd, (G) αN1+αN2, (H) αN1 or (I) αN2 (mean±SEM, n=3 independent patient-derived organoid lines). Scale bar: 250 μm. ND, not detected.