THE EMBO JOURNAL

Manuscript EMBO-2014-90583

Notch Signaling Regulates Gastric Antral LGR5 Stem Cell Function

Elise S Demitrack, Gail B Gifford, Theresa M Keeley, Alexis J Carulli, Kelli VanDussen, Dafydd Thomas, Thomas J Giordano, Zhenyi Liu, Raphael Kopan and Linda C Samuelson

Corresponding author: Linda Samuelson, University of Michigan

Review timeline:

Submission date:	17 November 2014
Editorial Decision:	19 December 2014
Revision received:	30 June 2015
Editorial Decision:	16 July 2015
Accepted:	16 July 2015

Editor: Thomas Schwarz-Romond

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 December 2014

Thank you very much for submitting your paper on Notch-dependent proliferation of gastric stem cells for consideration to The EMBO Journal editorial office.

You will find comments of three scientists below that indicate potential general interest in your findings.

However, both ref#2 and #3 take issue with:

(i) the conclusion that Notch-functions on Lgr5+ stem cells, as this seems not sufficiently supported by the currently presented experimental evidence.

(ii) Further, the involvement of TOR-signaling/its causal interlink with Notch, while truly intriguing, seems by far not convincing and would need much stronger molecular and functional corroboration

before the paper could be further considered for presentation at the level of The EMBO Journal.

These very explicit requests are truly demanding and need significant further experimental repetitions and expansions. Given the general interest in your proposal however, I would not be opposed to give you a chance to pursue one round of significant revisions.

I do urge you however, to consider your options at this point very carefully, also to avoid irritations/disappointments much later in the process and in case you prefer more rapid publication of at a less stringent publication, instead of having to invest valuable time, resources and efforts in

the attempt to formally revise for The EMBO Journal.

Please do not hesitate to get in touch with regard to feasibility and anticipated timeline for the necessary revisions (due to time constrains preferably via e-mail) OR in case you may need more time than the standard three month for formal revisions.

I hope that this decision will be perceived as reasonable and rational.

I also take the liberty to wish you an enjoyable festive season in any case and would be happy to hear from you on prefer to proceed.

REFEREE REPORTS

Referee #1:

This is a very thorough, well performed study of the role of Notch signalling in the stomach. There appear to be clear differences from the small and large intestines. I have no major criticisms and would simply like to see more evidence that the intestinal phenotypes of the mice are those expected.

Referee #2:

In the manuscript "Notch Signalling Promotes Proliferation of LGR5+ Gastric Antral Stem Cells via activation of mTOR" by Elise S. Demitrack et al. the authors describe how Notch signalling is involved in stem cell proliferation and differentiation of the mouse stomach antrum epithelium. The authors investigated the effect of Notch signalling inhibition and activation in the adult mouse stomach and found that inhibition of Notch signalling by DBZ results in a reduction of proliferative LGR5+ stem cells in vivo and a reduced organoid formation efficiency in vitro. In contrast, activation of Notch signalling resulted in increased proliferation in vivo and increased plating efficiency in vitro. Notch activation also resulted in an increase in the percentage of gland fission events. Additionally the authors showed that Notch inhibition facilitates differentiation whereas Notch activation leads to reduced differentiation. Confetti lineage tracing analysis revealed that activation of Notch signalling promotes faster clonal expansion of Lgr5+ stem cell clones. Long term activation of Notch signalling resulted in polyp formation which showed high proliferation and high expression of stem/progenitor cell marker while harbouring only small numbers of fully differentiated cells. The authors showed that Notch signalling mediated increase of proliferation and gland fission can at least partly be rescued by inhibition of mTOR signalling, providing a functional link between these two pathways in the stomach antrum epithelium. This is an interesting study about the role of the Notch signalling pathway in the stomach antrum. However, there are several issues that need to be resolved:
Major concerns:
1. In figure 2 the authors clearly show the effect of Notch inhibition and activation on stem cell proliferation and on the Notch target Olfm4. Additionally to this information it would be crucial to quantify the number of Lgr5+ stem cells under both conditions to evaluate the effect of Notch inhibition/activation on the stem cell compartment by Lgr5 GFP and/or by in situ hybridisation. This should also be performed for polyps formed after constitutive Notch activation.
2. In figure 3 the authors analyse the replating efficiency of organoids with and without inhibition of Notch signalling. In this case the authors measured a "fold change vs. pre passage" presumably counting the number of organoids before and after passaging. Due to the reduced organoid size (probably reduced number of LGR5+ stem cells) after Notch inhibition it would be recommended to quantify the replating efficiency by sorting Lgr5+ stem cells after DAPT treatment. This would allow plating of same numbers of Lgr5+ stem cells. The replating efficiency would then be evaluated by measuring the organoid forming efficiency under these conditions.
3. In figure 6 the authors show a very interesting analysis of clonal competition and increased gland cluster formation as a result of Notch activation. However, providing only one single time point makes it very difficult to interpret the data. Therefore it is recommended to show at least three different time points after activation of Notch signalling to illustrate the progression towards monoclonality and cluster formation.

The authors should also explain the scoring strategy of gland clusters in the main text (clustered adjacent glands expressing the same confetti colour). It is recommended to show a lower magnification image of the XZ-section to present a better overview of the clone distribution. An example of a gland cluster should be marked by a dotted line for clarification.

Minor points:
- In figure 1 K and L and figure 2 C and D the authors label the control as vehicle treated. If this is a labelling mistake, this should be corrected. The control should be "Lgr5" treated with the same Tamoxifen injection.
- For figure 2 it is advised to include representative images of GFP+ / Ki67+ cells in histological sections of control, DBZ treated and Lgr5;NICD mouse antrum.
- On page 5 and in Supplementary figure S1 the authors mention the use of a Bmi1 -CreERT2 mouse to delete RBPJ-κ. Given that Lgr5-CreERT2 lines have been used throughout the manuscript it should be mentioned if there was a particular reason for using Bmi1-CreERT2.
- On page 7 the authors refer to Fig 2H. The figure that is being referred to should be Fig. 2J?
- In Figure 8 the authors clearly illustrate the strong expression of CD44 and Sox9 in antral polyps. In the intestine these are Wnt target genes. Therefore it is advised to analyse Wnt activity by performing β-Catenin staining (Cytoplasmic/Nuclear?)
- Figure 8 panels G and H are not essential for the main message of Fig 8 and should be moved to supplementary data.
- In Figure9 G and J it would be helpful for the reader to include data of untreated control mice (Lgr5) to show to what extend Rapamycin can rescue the effect of Notch activation.
- In Supplementary figure 4 the authors show that Rapamycin treatment does not affect Notch signalling. Though this is getting very clear, the panels C, D, G and H show unexpected nuclear GFP signal in addition to the cytoplasmic GFP in the base of antral glands. The authors should describe what kind of labelling/staining is shown in these panels.

Referee #3:

In this manuscript, the authors demonstrated that genetic Notch activation induces proliferation and disrupts differentiation of gastric epithelial cells, and further induce gland fission and antral polyps, while notch inhibition inhibits proliferation of the tissue. It has been demonstrated in previous publications that Notch inhibition induces Lgr5+ intestinal stem cell differentiation towards secretory lineages and Notch activation is necessary for stem cell maintenance and proliferation. It will be interesting to study whether similar effects are shown in Lgr5+ stem cells in the stomach. However, there are some concerns that should be addressed in the manuscript:

Major concerns:

1. The major issue is most of the analysis was performed on antral epithelial tissue (containing mixed population of cells in addition to the stem cells) rather than directly on Lgr5+ stem cells, although the authors claimed so. Because the most profound effects of Notch activation/inhibition seem to be on the transit amplifying cells rather than Lgr5+ stem cells in the tissue, detailed analysis should be performed directly on Lgr5+ stem cells. i.e. on the maintenance, proliferation and differentiation of Lgr5+ stem cells.

Specifically, in figure 2A,C, the number of Lgr5+ cells (i.e. GFP+ cells) could also be shown for comparison. In figure 2E-L, the plating efficiency should also be tested directly on Lgr5+ stem cells (i.e. single Lgr5+ stem cells isolated from mice with different treatment). In Figure 3, the maintenance of Lgr5+ stem cells should be analyzed by measuring GFP expression. It has been previously shown that Notch activation by VPA maintains Lgr5-GFP expression in Lgr5+ intestinal stem cells and gastric stem cells and Notch inhibition induces in vitro differentiation of the stem cells (Yin et al., 2014, Nature Methods). Will similar results be obtained by force expression of NICD or DBZ treatment? In addition, the differentiation of cells in organoids should also be analyzed to further support the claim that Notch activation inhibits differentiation of Lgr5+ stem cells.

2. The authors claim that Notch regulates stem cell proliferation via mTOR signaling. This conclusion is not well supported. The authors should perform more experiments to support this or

remove this conclusion.
Specifically, it's not clear whether this effect truly starts in Lgr5+ stem cells or in more differentiated cells. In figure 9A-D, the expression of pS6 did not co-localize in stem cell region, and it's not clear whether Lgr5+ stem cells have (or increased/decreased) pS6 expression.

In addition, in figure 9E-J, the effects that mTOR inhibition attenuated the increased proliferation and gland fission could also be explained that mTOR and Notch are both needed for stem cell activity, but with separate functions. In Supp.Fig.4, suppose that Notch is active in basal epithelial cells, if Notch really effects via mTOR, then why these cells are not affected following rapamycin treatment.

Other concerns:
3. It is necessary to show the efficiency of NICD induction or expression in the tissue post-TX.

4. For the experiments with monoclonal conversion (Figure 6), it will be interesting to show antral gland at early time points (before monoclonal conversion) and show the efficiency of NICD induction (and possibly expression of NICD in stem cells with different colors). This will help reveal the mechanism of increased monoclonal conversion rate: is it because NICD expression in some Lgr5+ stem cells provides them growth advantage over other non-NICD expression cells in the same gland or because of other mechanisms?

5. Figure 6. It will be helpful to show representative images for the "gland clusters".

1st Revision - authors' response 30 June 2015

Reviewer #1:

This is a very thorough, well performed study of the role of Notch signalling in the stomach. There appear to be clear differences from the small and large intestines. I have no major criticisms and would simply like to see more evidence that the intestinal phenotypes of the mice are those expected.

We thank the reviewer for the positive comments about our manuscript. As requested we now include data showing that Notch activation in intestinal Lgr5+ stem cells resulted in expected changes to cellular differentiation. At 1-month post-tamoxifen treatment of *Lgr5; ROSA*^{NICD} mice we observed crypt/villus units expressing NICD-GFP that were largely devoid of goblet cells (data added to Figure EV3). A loss of goblet cells with Notch activation is consistent with a previous report of NICD activation in adult intestine (Stanger et al. 2005), which follows the known role of Notch signaling to inhibit secretory cell differentiation. Note that the cellular changes resulting from NICD activation are patchy due to the mosaic expression pattern of the Cre recombinase in the Lgr5-GFP-CreER^{T2} mice.

With respect to the Notch inhibition studies, we previously reported an extensive analysis of intestinal stem cell proliferation and differentiation in DBZ-treated mice (VanDussen et al. 2012). The intestine of the DBZ-treated mice showed extensive secretory cell hyperplasia. The stomachs from those mice were analyzed and that data was included in the current manuscript. We have added a comment in the Methods section to indicate that the DBZ-treated intestine had the expected cellular changes, as reported previously (VanDussen et al. 2012).

Thus, the expected intestinal phenotypes were observed in the mice used for this study at early time points. However, when the Notch-activated mice were analyzed at later time points we did not observe intestinal phenotypes. We attribute the lack of intestinal polyps (Fig EV3C-J), as well as unchanged proliferation (new data added to Fig EV3K&L), stem cell marker and Notch target gene expression (new data added to Fig EV3M&N) in *Lgr5; ROSA*^{NICD} mice 6-months post-tamoxifen to be due to the inefficient recombination induced by the Lgr5-Cre driver and eventual loss of the NICD-activated stem cells over time. However, a detailed analysis of the NICD-activated intestine to understand the mechanism underlying the different outcomes relative to the NICD-activated stomach is outside of the scope of the current study.

Reviewer #2:

Major Comments:

1. In figure 2 the authors clearly show the effect of Notch inhibition and activation on stem cell proliferation and on the Notch target Olfm4. Additionally to this information it would be crucial to quantify the number of Lgr5+ stem cells under both conditions to evaluate the effect of Notch inhibition/activation on the stem cell compartment by Lgr5 GFP and/or by in situ hybridisation. This should also be performed for polyps formed after constitutive Notch activation.

We thank the reviewer for this suggestion. As requested we performed *in situ* hybridization for Lgr5 and interestingly saw a marked reduction in expression of this stem cell marker in the polyps (Figure EV4). We found that although hyperproliferative and undifferentiated, the polyps are almost completely devoid of Lgr5 expression (EV4 panel J). This finding was also supported by the lack of Lgr5-GFP expression in the polyps and the lack of Confetti lineage re-tracing after tamoxifen re-treatment of *Lgr5; ROSA^{Con}; ROSA^{NICD}* mice six months after NICD activation (EV4 panels D-G). Furthermore, in response to point 5 below, we observed reduced Wnt target gene expression in Notch-activated tissues and organoids. Thus, because Lgr5 is a Wnt target gene, this finding complicates using Lgr5 as a stem cell marker in some of our experiments. Moreover, as discussed above, use of GFP to count Lgr5-GFP stem cells is not possible in the Notch-activated (*Lgr5; ROSA^{NICD}*) mice because GFP is included in the NICD construct, which precludes the use of the GFP marker to identify Lgr5-GFP stem cells by FACS analysis.

As described above we have focused our analysis of Notch effects using functional measurement of stem cells by efficiency of organoid formation and lineage tracing activity. These functional studies have shown consistent changes in stem cell activity, with reduced activity with Notch inhibition and increased activity with Notch activation. Because we do not have direct measurement of stem cell numbers we have taken these comments out of the text and now refer to stem cell activity or function rather than stem cell number.

2. In figure 3 the authors analyse the replating efficiency of organoids with and without inhibition of Notch signalling. In this case the authors measured a "fold change vs. pre passage" presumably counting the number of organoids before and after passaging. Due to the reduced organoid size (probably reduced number of LGR5+ stem cells) after Notch inhibition it would be recommended to quantify the replating efficiency by sorting Lgr5+ stem cells after DAPT treatment. This would allow plating of same numbers of Lgr5+ stem cells. The replating efficiency would then be evaluated by measuring the organoid forming efficiency under these conditions.

We thank the reviewer for this interesting suggestion. Unfortunately we were unable to obtain sufficient numbers of Lgr5^{+} stem cells from the organoids to perform this experiment. Thus to address this point we sorted single Lgr5-GFP cells from vehicle or DBZ-treated mice, and found that Notch inhibition led to a reduced efficiency of organoid establishment from equal numbers of single Lgr5+ stem cells. These data along with representative images of the organoids have been added to Figure 2 as new panels (M-O).

3. In figure 6 the authors show a very interesting analysis of clonal competition and increased gland cluster formation as a result of Notch activation. However, providing only one single time point makes it very difficult to interpret the data. Therefore it is recommended to show at least three different time points after activation of Notch signalling to illustrate the progression towards monoclonality and cluster formation.

We thank the reviewer for this suggestion, which has allowed us to expand our understanding of the process of stem cell function after Notch activation. As requested, we added data from two additional time points, now showing Confetti tracing data from 2, 5 and 8 weeks after Notch activation. This analysis showed that monoclonal gland labeling was extremely rapid in *Lgr5; NICD* mice, with extensive single-color labeling apparent as early as two weeks post-tamoxifen treatment. Furthermore, this analysis showed a progressive increase in the number of clusters of same-colored adjacent glands after NICD activation, which is consistent with our conclusion that Notch activation induces gland fission. These data are discussed in the Results section (page 10) and included in Figure 6 and Appendix Figure S2.

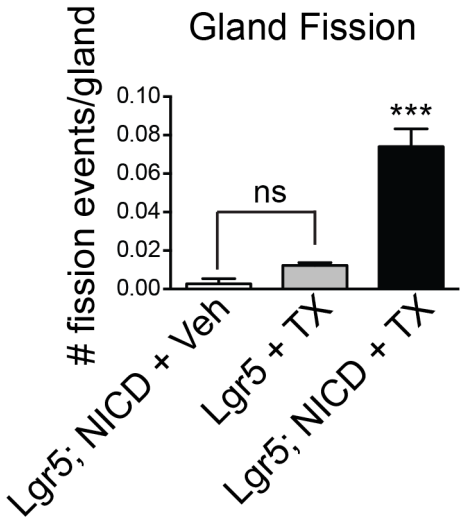
The authors should also explain the scoring strategy of gland clusters in the main text (clustered adjacent glands expressing the same confetti colour). It is recommended to show a lower magnification image of the XZ-section to present a better overview of the clone distribution. An example of a gland cluster should be marked by a dotted line for clarification.

As requested, the gland cluster scoring strategy is explained in the main text (page 10) and examples of gland clusters are marked in Figure 6, panel D and in Appendix Figure S2, panel D. Lower magnification images of XZ-sections from TX-treated control (*Lgr5*) and *Lgr5; NICD* mice 8-weeks post-TX are shown in Appendix Figure S2, panels C&D.

Minor Comments:

1. In figure 1 K and L and figure 2 C and D the authors label the control as vehicle treated. If this is a labelling mistake, this should be corrected. The control should be "Lgr5" treated with the same Tamoxifen injection.

For these experiments, the control groups were vehicle-injected mice of the same genotype. In other experiments we have analyzed tamoxifen-treated Lgr5 mice and did not observe a significant difference from vehicle-treated *Lgr5; NICD* mice. This comparison is provided below for the gland fission data included in Figure 5.



Furthermore, for the 6-month NICD studies, all controls were Lgr5 mice injected with tamoxifen and antral polyps were never observed. We attribute our NICD phenotype purely to effects of chronic Notch activation.

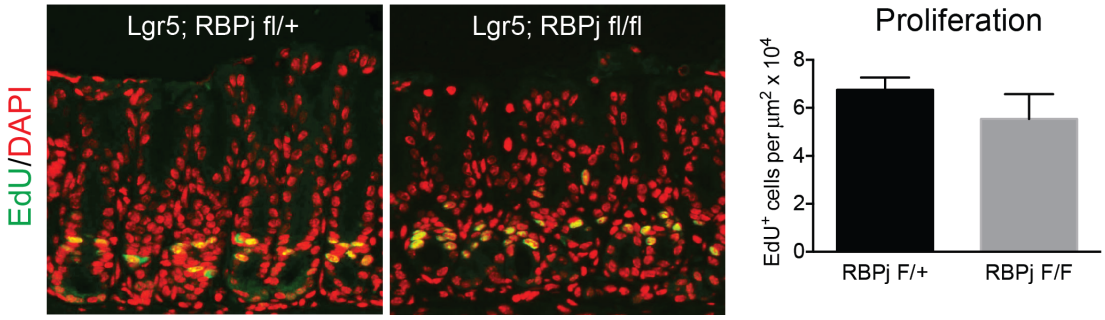
2. For figure 2 it is advised to include representative images of GFP+ / Ki67+ cells in histological sections of control, DBZ treated and Lgr5;NICD mouse antrum.

Examples of GFP+/Ki67+ cells from control, DBZ and 1-mo *Lgr5; NICD* are now included in Appendix Figure S1.

3. On page 5 and in Supplementary figure S1 the authors mention the use of a Bmi1 -CreERT2 mouse to delete RBPJ-κ. Given that Lgr5-CreERT2 lines have been used throughout the manuscript it should be mentioned if there was a particular reason for using Bmi1-CreERT2.

Bmi1-CreER was utilized to delete RBPJ-κ because it is more uniformly expressed in the antrum than the patchy Lgr5-GFP-CreER^{T2} (Samuelson laboratory unpublished data). Analysis of Lgr5; RBPj$^{fl/+}$ (n=3) and Lgr5; RBPj$^{fl/fl}$ (n=3) mice using an identical tamoxifen regimen and tissue harvest (5 days 100 mg/kg TX, harvest day 6) showed no effect on proliferation in the antrum. Those results are included below. Our interpretation of these negative data is that

LGR5^{+} antral stem cells with RBPj-κ deletion are lost and replaced by non-recombined (or "normal") stem cells.

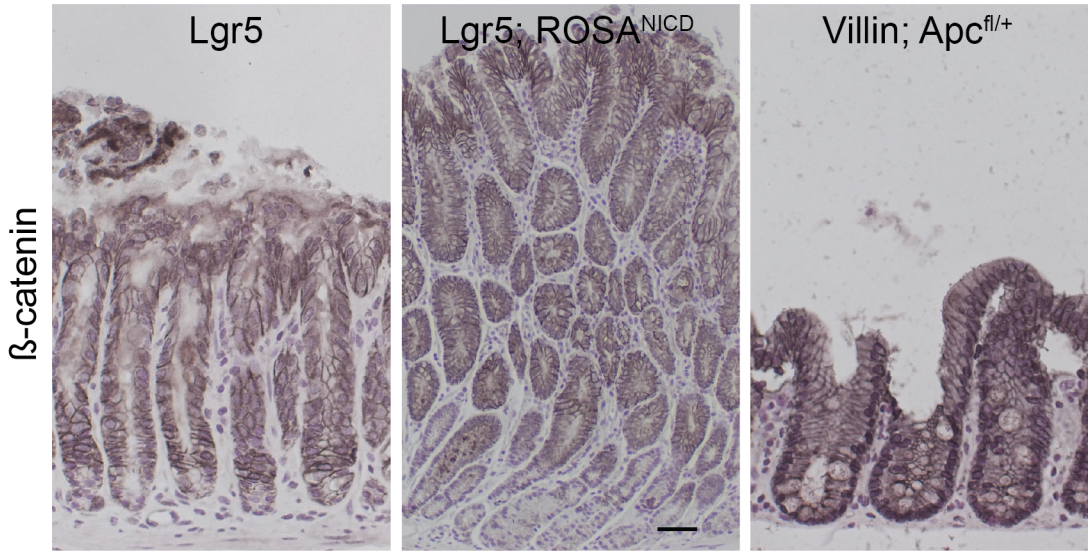


4. On page 7 the authors refer to Fig 2H. The figure that is being referred to should be Fig. 2J?

We thank the reviewer for catching our error. This mistake has been corrected.

5. In Figure 8 the authors clearly illustrate the strong expression of CD44 and Sox9 in antral polyps. In the intestine these are Wnt target genes. Therefore it is advised to analyse Wnt activity by performing β-Catenin staining (Cytoplasmic/Nuclear?)

We thank the reviewer for this suggestion. We have performed an analysis of Wnt activity in *Lgr5; ROSA^{NICD}* polyps (qRT-PCR for Axin2 and Lgr5, β-catenin IHC), as well as performed additional experiments to assess Lgr5 expression in organoids and *Lgr5; ROSA^{NICD}* mice 6-mos and 1-yr post-tamoxifen treatment. These data have been compiled in a new Expanded View figure (Figure EV4). We found no evidence that the Wnt pathway is activated in *Lgr5; ROSA^{NICD}* mice; on the contrary, we found that Notch activation led to a significant reduction in expression of Wnt target genes. Furthermore, loss of Lgr5 in NICD polyps was confirmed by GFP immunostaining for Lgr5-GFP marked cells (Fig EV4E), *in situ* hybridization (Fig EV4J), and a lack of Confetti re-tracing in *Lgr5; ROSA^{NICD}* polyps (Fig EV4J). These findings agree with a recent publication suggesting a role for Notch in suppressing Wnt signaling in the intestine (Tian et al. 2015). We did not add the β-catenin IHC to Figure EV4 because it did not add additional information. However this staining, along with nuclear β-catenin staining in the Vilin-Cre; Apc$^{fl/+}$ mouse colon as a positive control, is included below for the reviewer.



6. Figure 8 panels G and H are not essential for the main message of Fig 8 and should be moved to supplementary data.

Figures 8G and 8H have now been moved to Appendix Figure S2.

7. In Figure9 G and J it would be helpful for the reader to include data of untreated control mice (Lgr5) to show to what extend Rapamycin can rescue the effect of Notch activation.

We thank the reviewer for this suggestion as the results from this experiment further solidified our conclusion that the mTOR pathway plays a functional role in mediating NICD-induced proliferation and gland fission. We have quantified proliferation (Figure 9G) and antral gland fission (Figure 9H) in untreated C57BL/6 mice and added those data as a 3rd group to each respective graph. These data lead us to conclude that rapamycin treatment of *Lgr5; ROSA^{NICD}* mice returns proliferation and gland fission to basal levels.

8. In Supplementary figure 4 the authors show that Rapamycin treatment does not affect Notch signalling. Though this is getting very clear, the panels C, D, G and H show unexpected nuclear GFP signal in addition to the cytoplasmic GFP in the base of antral glands. The authors should describe what kind of labelling/staining is shown in these panels.

We apologize for not being clear with the labeling of this figure. The *ROSA^{NICD}* construct contains a nuclear GFP sequence that allows for detection of the NICD transgene via GFP immunostaining. We have clarified the description of these panels in the legend for this figure, which is now renamed Figure EV5.

Reviewer #3:

Major Comments:

1. The major issue is most of the analysis was performed on antral epithelial tissue (containing mixed population of cells in addition to the stem cells) rather than directly on Lgr5+ stem cells, although the authors claimed so. Because the most profound effects of Notch activation/inhibition seem to be on the transit amplifying cells rather than Lgr5+ stem cells in the tissue, detailed analysis should be performed directly on Lgr5+ stem cells. i.e. on the maintenance, proliferation and differentiation of Lgr5+ stem cells.

Specifically, in figure 2A,C, the number of Lgr5+ cells (i.e. GFP+ cells) could also be shown for comparison. In figure 2E-L, the plating efficiency should also be tested directly on Lgr5+ stem cells (i.e. single Lgr5+ stem cells isolated from mice with different treatment).

As suggested by the review, we performed single Lgr5-GFP stem cell sorting from vehicle and DBZ-treated mice and found a reduction in organoid-forming efficiency from Notch-inhibited stem cells. This is an important finding that shows direct effects on Lgr5 stem cells, and we have focused our conclusions regarding Notch regulation of antral stem cells on stem cell activity/function rather than stem cell number. These data have been added as new panels in Figure 2 (Figure 2M-O). As stated above, we were unable to perform parallel experiments with the Notch activated model due to the nuclear GFP sequence in the ROSA^{NICD} allele, which precluded sorting Lgr5-GFP stem cells from *Lgr5; ROSA^{NICD}* mice.

In Figure 3, the maintenance of Lgr5+ stem cells should be analyzed by measuring GFP expression. It has been previously shown that Notch activation by VPA maintains Lgr5-GFP expression in Lgr5+ intestinal stem cells and gastric stem cells and Notch inhibition induces in vitro differentiation of the stem cells (Yin et al., 2014, Nature Methods). Will similar results be obtained by force expression of NICD or DBZ treatment?

To examine the effect of Notch signaling on stem cells we measured Lgr5 mRNA abundance in the organoid models. We found that Lgr5 expression was not different in Notch-inhibited organoids, but was significantly reduced in Notch-activated organoids (Figure EV4C). As explained in our response to Reviewer #2 (minor comment #5), we also observed a reduction in Lgr5 expression in *Lgr5; ROSA^{NICD}* mice. Our findings suggest that Notch activation may suppress Wnt signaling and Lgr5 expression.

In addition, the differentiation of cells in organoids should also be analyzed to further support the claim that Notch activation inhibits differentiation of Lgr5+ stem cells.

As requested, we measured differentiation in Notch-inhibited (DAPT-treated) and Notch-activated (*Lgr5; ROSA*^{NICD}) organoids by qRT-PCR analysis of markers of endocrine (Gastrin and Ngn3) and deep mucous (Tff2 and Spdef) cells. The findings agreed with our *in vivo* results, showing that Notch inhibition induced differentiation and Notch activation suppressed differentiation. These data have been added as new panels to Figure 4 (Figure 4P-S).

2. The authors claim that Notch regulates stem cell proliferation via mTOR signaling. This conclusion is not well supported. The authors should perform more experiments to support this or remove this conclusion. Specifically, it's not clear whether this effect truly starts in Lgr5+ stem cells or in more differentiated cells. In figure 9A-D, the expression of pS6 did not co-localize in stem cell region, and it's not clear whether Lgr5+ stem cells have (or increased/decreased) pS6 expression.

In addition, in figure 9E-J, the effects that mTOR inhibition attenuated the increased proliferation and gland fission could also be explained that mTOR and Notch are both needed for stem cell activity, but with separate functions. In Supp.Fig.4, suppose that Notch is active in basal epithelial cells, if Notch really effects via mTOR, then why these cells are not affected following rapamycin treatment.

We thank the reviewer for this comment. As detailed above in our comments to the editor, we acknowledge that we over-stated our original conclusion regarding Notch regulation of stem cell proliferation and gland fission as occurring via the mTOR signaling pathway. Thus we have removed these statements from the revised manuscript. We maintained our interesting data showing that mTORC1 signaling is required for the NICD-induced increases in proliferation and gland fission, but do not make global conclusions about the mechanism of Notch and mTOR interaction. It is interesting that rapamycin treatment does not appear to affect normal tissue homeostasis or proliferation (Figure EV5). Thus mTORC1 does not appear to be an essential downstream mediator of Notch function in the normal gastric antrum.

Although the pS6 staining is not apparent in the stem cell zone, a recent study showed that pS6 staining in the intestinal crypt was dependent on feeding status, with no staining observed in fasted mice and abundant staining observed after re-feeding (Yilmaz et al. 2012). All of our tissues are collected from fasted mice, which may explain the lack of staining in the progenitor zone of the stomach.

Other Comments:

3. It is necessary to show the efficiency of NICD induction or expression in the tissue post-TX.

We have quantified NICD$^+$ glands 1 month post-TX treatment and found that 57% of antral glands are NICD$^+$ at this timepoint. This information was added to the text on page 6.

4. For the experiments with monoclonal conversion (Figure 6), it will be interesting to show antral gland at early time points (before monoclonal conversion) and show the efficiency of NICD induction (and possibly expression of NICD in stem cells with different colors). This will help reveal the mechanism of increased monoclonal conversion rate: is it because NICD expression in some Lgr5+ stem cells provides them growth advantage over other non-NICD expression cells in the same gland or because of other mechanisms?

As discussed above for Reviewer #2, point 3, we extended this analysis to include two earlier time points. We detected extensive single-colored glands as early as 2-weeks after NICD activation, suggesting rapid spread of Notch-activated stem cells within the stem cell niche. These data are discussed in the Results section (page 10) and included in Figure 6 and Appendix Figure S2.

It is not possible to follow NICD in stem cells with different colors due to the overlapping staining expected from the nuclear GFP in the NICD allele and the nuclear GFP in the Confetti allele.

5. *Figure 6. It will be helpful to show representative images for the "gland clusters".*

As stated above in our response to Reviewer 2, we have now outlined representative gland clusters in Figure 6 and Appendix Figure S2. Additionally, low magnification XZ images from *Lgr5* and *Lgr5; NICD* mice 8 weeks post-TX have been added to Appendix Figure S2, which better illustrates the distribution of polyclonal, monoclonal and clustered monoclonal glands.

References

Stanger BZ, Datar R, Murtaugh LC, Melton DA. 2005. Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci U S A* **102**: 12443-12448.

Tian H, Biehs B, Chiu C, Siebel CW, Wu Y, Costa M, de Sauvage FJ, Klein OD. 2015. Opposing activities of Notch and Wnt signaling regulate intestinal stem cells and gut homeostasis. *Cell Rep* **11**: 33-42.

VanDussen KL, Carulli AJ, Keeley TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kolterud A et al. 2012. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. *Development* **139**: 488-497.

Yilmaz OH, Katajisto P, Lamming DW, Gultekin Y, Bauer-Rowe KE, Sengupta S, Birsoy K, Dursun A, Yilmaz VO, Selig M et al. 2012. mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. *Nature* **486**: 490-495.

2nd Editorial Decision 16 July 2015

Thank you for submitting your revised manuscript. Your manuscript has until now been handled by my colleague Thomas Schwarz-Romond, but as he is no longer with The EMBO Journal I have stepped in as 2nd editor on the manuscript. I have sent your revised manuscript back to referees #2 and 3 and I have now received their comments back.

As you can see below, the referees appreciate the introduced changes and support publication here. Referee #3 suggests including two additional references. Both references seem relevant, but I will leave that up to you.

REFEREE REPORTS

Referee #2:

The revised version has addressed all my concerns and became much clear and solid. I fully recommend it for publication.

Referee #3:

Authors should cite,

Tien et. al. Cell Rep. 2015 Apr 7;11(1):33-42.

Yin et. al Nat Methods. 2014 Jan;11(1):106-12.

Author have addressed all of my feedback and the manuscript is suitable for publication in EMBO Journal.

