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Gastric stem cells promote inflammation and gland remodeling in response to H. pylori via Rspo3-Lgr4 axis

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Sigal,

Thank you again for the submission of your manuscript (EMBOJ-2021-109996) to The EMBO Journal and in addition providing us with a preliminary revision plan. As mentioned earlier, your study has been sent to three reviewers for evaluation, and we have received reports form all of them, which I enclose below.

As you will see from their comments, the referees acknowledge the potential interest and value of your findings, although they also express major concerns. In more detail, referee #2 states substantial issues regarding the mechanistic depth and conceptual insights provided into a role of Rspo3-Lgr4-NFkB axis in H. pylori-driven stomach epithelial reprogramming. Also, this reviewer points to major concerns on potential confounding effects of Lgr5 alterations in the Lgr4 genetic loss-of-function model, which in his-her view diminishes the relevance of the current analysis. Referee #3 agrees in that the impact of Lgr4 loss on Lgr5 impacts on conclusions about differential receptor function in this context (ref#3, pt.1). Also, this expert is concerned about unresolved potential confounding roles of genetic background and mouse strains (ref#3, pt.2).

Given the interest stated and broader angle of your findings, we are overall able to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline. I need to stress though that we do require strong support from the referees on a revised version of the study in order to move on to publication of the work. As to the open outcome of the revisional work I suggest keeping EMBO Reports in mind for this study as an alternative venue.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
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7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be

provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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Thank you for the opportunity to consider your work for publication.
I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 16th Feb 2022:

Link Not Available

Referee #1:

The manuscript by Wizenty J et al, entitled "Antrum stem cells promote inflammation and gland remodeling upon exposures to H.pylori", describes some interesting and useful observations regarding the role of Lgr4+ and Lgr5+ antral crypt progenitors in the response of the gastric mucosa to H. pylori infection. In previous work from this lab, Dr. Sigal first described the role of stromal R-spo3 signaling on aAxin+Lgr5- antral stem cells during homeostasis and H. pylori infection (Sigal M et al, Nature 2017, and then went on to show that upregulation of Rspo3 by H. pylori infection leads to secretory differentiation of Lgr5+ cells which contribute to host defense (Sigal M et al, Nat Cell Biol 2019). In the current submission, the authors analyze in more depth the involvement of Lgr4 and Lgr5 receptors in regulating gland remodeling upon H. pylori infection, with a novel link shown between Rspo and NF-kB signaling. Overall, the data are of interest and provide convincing evidences on the requirement of Lgr4 receptor, but not Lgr5, in directing the H. pylori response in the gastric antrum. Given the limited data to date on Lgr4+ cells (in contrast to voluminous reports on Lgr5+ cells), this report fills an important gap and will be valuable to the field. However, the study nevertheless has a number of weaknesses and limitations, as outlined below, and I do have some concerns in regards to the interpretation of the results and conclusions drawn.
Major concerns:
1. Introduction of Lgr4. Perhaps more rationale should be provided in the Introduction regarding why Lgr4 is studied here. In fact, the two previous papers pointed to the Axin2+Lgr5- cells in the gastric isthmus as mediating responses to Rspo3. And the one in situ hybridization figure from the 2017 paper (Extended Data Fig. 8d) did show expression of Lgr4, but not Lgr5 or Lgr6, in this isthmal region. However, it is likely a bit misleading to state that "Lgr4 is expressed throughout the gland" as sort of implies that Lgr4 is expressed ubiquitously in every antral epithelial cells, which is probably not the case. Has Lgr4 expression been further studied in single cell studies or CreERT mapping or any other technique. Perhaps better to say that Lgr4 is more broadly expressed above the antral gland base with a concentration in the antral isthmus region.
2. Characterization of conditional knockout of Lgr4 and Lgr5. The analysis and immunofluorescent staining of antral glands from the Axin2-CreERT conditional knockout mice is shown in Supplementary Figure 1. However, while it is stated correctly that there were "no major pathologies", the analysis is a bit unsatisfying as the reason for the particular stains is not well explained and the images a bit hard to appreciate. Perhaps it should be clarified once again from the Sigal et al 2019 study that the basal antral cells are secretory mucous cells that are derived from (and may overlap with) basal Lgr5+ cells, and that they differentiate in an Rspo3 dependent manner. It was previously shown I think that lectin binding with GSII and expression of GIF overlap a bit with the Lgr5+ cells at the base (See Sigal et al 2019, Fig. 1c, Fig 2e, f). In the gastric corpus, GSII+ cells are distinct from GIF+ cells, although a subset of secretory progenitors are positive for both (i.e. rare GSII+GIF+ cells). Are there in fact double positive GSI++GIF+ cells in the antrum?
3. With conditional knockouts, the authors suggest no change at baseline in "distribution" of GSII+ and GIF+ cells in the antrum, but their numbers appear a bit lower with knockout of Lgr4 and Lgr5. Quantification of "GSI+ area" shows a trend, but GIF staining is not quantified needs to be. PCR suggests that gene expression of GIF is decreased in both cases. Other genes that could be studied by PCR include TFF2 and MUC6 (which is measured in Fig. 1). What about expression of pit cell markers such as MUC5AC? Ki67 is stained for and appears lower in Lgr4F/F mice but is not quantified, and should be done in all four lines. Further, only one time point (day 14) is examined - was this phenotype stable with longer followup? At say 1-3 months? Given the requirement of Rspo signaling for progenitor cell function, it would be surprising to show that loss of Rspo receptors had no longer term consequences. Can antral 3D spheroids be grown from Lgr5fl/fl or Lgr4flf/fl mice? Finally, the most notable finding is that conditional knockout of Lgr4 leads to marked downregulation of Lgr5 expression. This needs perhaps a bit more explanation.
4. H. pylori infection. The point of the study is that much greater changes are seen in the conditional Lgr knockout mice after infection with H. pylori, given the normal response requires Rspo3 signaling. However, there are issues interpreting this data in that all the appropriate controls are not included. Thus, to interpret this data, we need to see both the Lgr5fl/fl infected and uninfected mice along with the corresponding infected and uninfected WT (i.e. Lgr5+/+) mice. There are in fact several comparisons that need to be made and we are only shown a few of them. Thus, there appears to be an expansion of GSII+ mucous cells in both the Lgr5+/+ and Fl/FL mice which is interesting, as this is seen in the corpus and has been called SPEM by some. GIF+ cells appear to be decreased, although they are not quantified (and should be). Finally, proliferation needs to be quantified. Most interesting is that there is no change in CFU with Lgr5 deletion, even though the previous study indicated that knockout of Rspo3 or Lgr5+ cells led to increased H. pylori colonization. Nevertheless, the overall conclusion seems right, which is that there is a more minimal effect with conditional Lgr5 gene deletion.
5. In contrast, more major changes were seen with conditional deletion of Lgr4 under conditions of H. pylori infection. There appears to be a significant loss of GSII+ cells and a loss of GIF+ cells. However, interpreting this is a bit tricky since data from uninfected Lgr4fl/fl is not shown. Most likely this reflects a lack of expansion of the GSII or GIF lineages, but in any case, the area of these lineages needs to be quantified, as does Ki67 staining which is likely decreased. Finally, the major finding is

apparently the change in colonization by H. pylori. Please introduce this as "H. pylori CFU" as CFU more often in Lgr5 papers refers to stem cell quantitation via growth in soft agar. In any case, conditional knockout of Lgr4 appears to lead to a marked increase in H. pylori colonization. However, it is odd that the control here has a different level of colonization than the control in Figure 1. In addition, if one "combines" the two figures, it is clear that there is no significant difference between Lgr5fl/fl and Lgr4fl/fl mice in their colonization, both being around 8 x 104. Some comment is needed - perhaps in the methods indicating the two groups were inoculated differently and that there is variability from experiment to experiment. In any case, given findings in Sigal et al 2019, this is hard to interpret as in that study, Lgr5 cells gave rise to the secretory lineage, but here Lgr4 cells are the origin of "gland base expansion." What is happening to Lgr5 cells here. The measure of Lgr5 gene expression is confusing, as the only comparison is Lgr4+/+ infection and Lgr4fl/fl infected and data presented as fold change with Lgr4+/+ infected included as the "control." In Supplementary Figure 1e, it was shown that at baseline, Lgr5 was expressed at much lower levels in uninfected Lgr4fl/fl mice compared to Lgr4+/+ mice. So is Lgr5 upregulated with H. pylori infection in Lgr4fl/fl mice, or is Lgr5 more downregulated in infected Lgr4+/+ mice? In the end, we need to see data from all 4 groups of mice to interpret the findings here.
6. Link between Rspo3 signaling and NF-kB pathways. Using whole transcriptome microarray, the authors observed a significant downregulation of multiple genes related to the NF-kB signaling in infected Lgr4fl/fl relative to WT controls. Using Myh11CreERT/Rspo3fl/fl mice, as well as in vitro antral organoids, they further analyze the relationship between R-spo3 and NF-kB signaling activation and conclude that Rspo3-Lgr4 axis is required to drive inflammatory response to H. Pylori. While the association between Rspo3-Lgr4 and NF-kB signaling appears evident, the proposed direct connection as well as the specificity of the cells involved is not strongly convincing or sufficiently fleshed out. First, while some NF-kB target genes are shown, the authors suggest that "most of the top downregulated genes were linked to innate immunity." How many genes were downregulated and how many were linked to innate immunity- presumably this was more than half and hopefully closer to two third. Perhaps a supplementary table with all of the genes listed and scored could be included. Second, through IHC of p65, the author suggested that NF-kB signaling activation to be restricted to the stem cell compartment under normal conditions. Immunofluorescence analysis for p65 (Fig. 6A) appears to show stronger signal intensity at the level of the isthmus, with broader labelling of the gland base can be observed in infected mice (Fig. 3G), but in both cases p65 seems to be absent from the most basal cells, which are typically occupied by the basal secretory cells. Thus, this data raises a number of questions that are not addressed. First, are the NF-kB regulated cytokines (Cxcl10, Ccl2, Cxcl1 and Cxcl2) expressed and secreted by antral epithelial cells? We as yet have no evidence for this, as the microarray analysis was performed on whole antral tissue. Second, if they are expressed by antral epithelial cells (and not the typical immune cells), are they expressed by the p65+ antral isthmus progenitors or by the GSII+ and GIF+ antral secretory cells? The authors suggest that NF-kB signaling is reduced in Lgr4fl/fl mice but no p65 staining is shown in these mice (on in the Rspo4fl/fl mice). Thus, where are Cxcl10, Ccl2, Cxcl1 and Cxcl2 produced?
7. 3D organoid experiments. Fig. 4 and 5. This presentation is more than a bit confusing. The author notes that organoids grown in full media (FM) have a baseline tone of NF-kB signaling higher than Wnt/Rspo free medium, based mainly on PCR measurements of cytokine genes. They then want to address the question as to why this does not seem to be the case in vivo upon single KO for Lgr5 or Lgr4. However, to start with, it is not clear that the authors have rigorously examined NF-kB signaling in the single knockout animals. In any case, the authors then proposes that baseline function can be compensated when only one receptor is present. Therefore, they go on generate double (Lgr4flf/fl;Lgr5fl/fl) knockout mice, which have reduced GSII+ area, and reduced GIF and PGC gene expression. In addition, they show significant downregulation of NF-kB genes. However, it is not clear that any of these findings represent significant differences from single knockout (e.g. Lgr4fl/fl) mice, which I would argue are nearly identical. Indeed, while the authors may want to raise the notion of compensatory effects by Lgr5 in the Lgr4fl/fl mice, in fact Lgr5 gene expression in Lgr4fl/fl mice is more than 80% decreased (Fig. S1E) and probably not much different than Lgr5 expression in the double knockout animals. However, apart from the actual level of expression, does Lgr5 expression change in location or expand beyond the crypt base in Lgr4fl/fl mice?
8. Inhibition of NF-kB signaling. Fig. 6. This is a useful experiment, with a dominant negative IkB, but this is not presented or explained well. Is this a constitutive IkB mouse, with inhibition in every cell type? It would be helpful to clarify just a bit more the features of this mouse. The finding of reduced glandular differentiation in vitro and in vivo, along with increased proliferation, is interesting and consistent, but still a bit unclear where this is actually occurring. The term "stem cell differentiation" is used here, but is this in the isthmus or more towards the gland base. What are the proliferating cells - are they Lgr4+? The data seem to support Lgr4+ cells as the relevant progenitors but then where do Lgr5+ cells fit in, since they appear to give rise to secretory cells in the previous paper (Sigal et al, 2019)? A bit more explanation would be helpful here. Perhaps a sentence or two could be added to the Discussion explaining the distinct roles of the Rspo3-Lgr4 and Rspo3-Lgr5 axis.

Minor concerns:
-The authors refer to the 129;129P2-ctnnb1tm(NFKBIAΔN)1Rsu mice as NF-kB-/-. While is understandable this choice the nomenclature used may be confusing and therefore a different nomenclature is recommended.
- The authors refer to "inflammation" frequently, but never really show (even in H. pylori-infected mice) an increase in leukocytes which is the definition of inflammation. Perhaps they should explain early on that by inflammatory responses, they mean the increase in proinflammatory cytokines.
-The authors show that constitutive inhibition of NF-kB signaling abrogates secretory cell specification in the mouse antrum. Is this effect specific of the mouse antrum or can be generalized to the gastric mucosa? What about the small intestine?
-While impeding secretory cell differentiation, NF-kB inhibition does not inhibit proliferation of isthmus progenitor cells and in fact accelerates it. Has the author tested if H. pylori infections can induce crypt hyperplasia in the mouse antrum independently of NF-kB activation?
-NF-kB inhibition strongly reduces Lgr5 expression in the mouse antral organoids (Fig. 6C). Is this true in vivo? Is Lgr4

expression altered in organoids or in this mice?
- In the discussion, the authors describe evidence that Rspo signaling drives expression of its receptor Lgr5, but no reference is provided. Please provide a reference to support this statement. Studies from Kuo CJ et al have shown in vivo increases in Lgr5-EGFP+ cells with Rspo treatment, but I'm not sure this is the same thing as receptor upregulation. In the same paragraph, I'm not sure what "constitutive expression of Lgr4" means.
- Subpopulation of H. pylori bacteria ... colonize the gastric glands. I'm not sure what is meant here. Do the authors mean that the bacteria colonizing the deep gastric glands are somehow different from the ones that colonize the pits? Or do they simply mean that a random subset of H. pylori bacteria somehow end up colonizing the gastric gland base. A few published studies have suggested that H. pylori can come into contact with antral stem cells. Perhaps these studies could be referenced.
-In the figures' legend (Figure 6) the author describe panel G and H. These panels are otherwise not referenced in the test and not present in the panel.

Referee #2:

Previously the senior author has shown that RSPO3 is important in the response to helicobacter infection in the mouse stomach. Here his group addresses which RSPO3 receptor, LGR4 or LGR5, is important. Lgr5 knockout with an Axin2 Cre has no obvious phenotype, while the Lgr4 ko fails to react to helicobacter infection. This implicates Lgr4, but there is a major problem with this interpretation noted below.

Looking at the consequences of Lgr4 knockout, they find these the Lgr4 ex/ex mice have apparently downregulated NF-κB signaling, a finding replicated by loss of the ligand RSPO3. This bit is strong and interesting but mechanistically remains a black box. In organoids they find that Wnt/RSPO signaling makes organoids permissive for expression of putative NF-κB targets using an interesting metabolite ADP-heptose and TNFa. Fig 5 detours a bit to show that gland base cells are altered in a formal Lgr4/5 double knockout, and show by microarray/GSEA that there is decrease in TNFa genes. Building a case that NF-κB is important in the stomach, they re-analyse a mouse with globally inhibited NF-κB and find alterations in a handful of gland base markers.

I found the flow of this manuscript hard to follow, and it took me several read-throughs to get the gist. I think this is because the strength of any specific conclusion is not very strong, as evidenced by the multiple instances of n=3 experiments and p values ~0.05. In addition, they tend to show many things by one experiment, e.g. a microarray and GSEA, and don't go deeper with confirmatory experiments. They go a long way on these modestly powered experiments, and there are multiple claims not supported by experiments. So while it's likely to be mostly correct and reproducible, the level of rigor is not high.

The biggest issue with the manuscript: The Lgr4fl/fl/Axin2CreErt2 mice after tamoxifen have greater loss of Lgr5 than Lgr4 mRNA. Suppl Fig 1E validates the knockout efficacy and introduces this issue, which is confirmed in Fig 2C. Please explain why Lgr4 fl/fl mice have more loss of Lgr5 mRNA than of Lgr4 mRNA? What is going on? Can they confirm genotyping? Did they lose expression of Wnt target genes?

This results makes separation of the role of Lgr4 versus Lgr5 a challenge that is not addressed. - it appears that Lgr4 ko acts like a double Lgr4 Lgr5 ko. So any phenotype seen in the Lgr4 ko is not cleanly an Lgr4 ko. Thus, a comment like "Thus, we conclude that Lgr4, but not Lgr5, is the critical receptor..." should perhaps read something like "Thus, we conclude that Lgr4 either alone or in combination with Lgr5, but not Lgr5 alone, is the critical receptor ..." "confirmed that Lgr4 depletion" -> "confirmed that Lgr4 plus Lgr5 depletion" etc. "response to H. pylori infection is driven by the Rspo3-Lgr4 axis" -> response to H. pylori infection is driven by the Rspo3-Lgr4/5 axis.

There is only one indication what the mouse background of the various strains, and that's a 129 NK-kb. Are the other mice B6 background? If so, how do they control for strain effects versus specific gene effects in B6 x 129 crosses? A full proper nomenclature (e.g. from Jax) would aid reproducibility. It's good practice to be very clear about genetic backgrounds, especially when there are multiple crosses.

Fig 3 - they assert than GSEA shows a decreased β-catenin signature, but no clear β-catenin target genes are shown in Fig 3B and the double KO mice (since the Lgr4 is effectively a double KO) don't have a proliferation phenotype. So I'm not convinced re: the β-catenin signature shown in 3A, unless this claim is supported with analysis of some specific β-catenin target genes. They could start with Axin2.

Isn't it surprising that the Lgr4 knockout (with low Lgr5) has no phenotype prior to infection? Loss of Lgr4/5 affects intestinal stem cells. Does it not have an effect on stomach? Please explain the background here.

The data on downregulation of NF-KB pathways is straightforward but I would like to know the status of the indicated genes in non-infected Lgr4/5 ko mice to know if this change is due to infection.

Since Lgr4/5 are the primary receptors for RSPO3, the data to this point confirm the prior reports on the importance of RSPO3

signaling and makes the new connection to NF-κB signaling.

Fig 4 shows that in organoids, Wnt signaling is required for NF-κB target expression. It would be useful to show in Fig 4C that the induction of target genes like Cxcl1 by ADP-heptose in this setting was indeed due to NF-κB rather than just assuming it. This could be done with small molecule inhibitors, and testing if NF-κB was indeed nuclear. The amount of data showing NF-κB itself is activated is limited to fig 3G.

While the authors assert on p. 6 that they "examine their ability to activate NF-κB via the ALPK1-TIFA-TRAF6 axis" the manuscript has no experimental data addressing the ALPK1-TIFA-TRAF6 axis. They should either include such experiments (a lot of work) or just mention this is one way it could happen.

(Here I'm again a bit confused - in Sigal 2019 depletion of Lgr5 expressing cells made stomachs permissive for helicobacter. How does that finding fit here?)

Fig 5 uses a genetic Lgr4/5 double knockout to show a modest depletion of gland base cells. I would expect a significant small intestine phenotype. Did that happen in their strain? They then assert a loss of tonic NF-κB signaling using only GSEA on microarray data but don't show any specific gene changes by PCR or at the protein level, nor do they show any biochemical evidence of decreased NF-κB signaling. This is a weakness.
Fig 6 could have addressed this but...They look at p65 protein abundance in normal stomach but not in the single or double knockouts. It's mostly in the cytoplasm, which I think means it's not signaling. They then use a mouse with presumed inactive NF-κB via constitutive expression of an active ikb (interestingly knocked into the β-catenin locus, making these mice haploinsufficient for β-catenin; Schmidt-Ullrich 2001) and find this has altered expression of Lgr5 (why?) and differentiation markers and Cxcl2 (why is Cxcl1 change in expression not significant?). This might be similar to what was observed in the Schmidt-Ullrich in other tissues. It's interesting that the phenotype is seen in Wnt-responsive tissues like hair follicles, teeth and gastric gland bases. Could this be related to the β-catenin haplo-insufficiency?

Other issues:
Please describe and/or reference the generation of the Lgr4 and Lgr5 floxed mice - there is no information here about where the loxP sites are, how they were generated, and how they were validated. There is only the insufficient statement "Lgr4fl/fl mice and Lgr5fl/fl mice were kindly provided by Dr. Jan Tchorz, Novartis." And given the problem with Suppl Fig 1E, how were they genotyped, and are they sure they didn't get the genotypes confused?

A bit of nomenclature confusion - Does Lgr5 fl/fl mouse refer to before or after tamoxifen? Or both? Normally, I think fl/fl refers to mouse with floxed but intact gene, and then you indicate the cre driver, and then ex/ex refers to after Cre-mediated excision if it's a conditional (e.g. CreER) excision. But that's just me. Please make it clearer. Using the fl/fl everywhere is not precise.

Pro tips: 1. Please number your pages. Numbering your lines would be even more helpful to the reviewer. I numbered the pages in my copy (using Acrobat) starting with the title page and may refer to those numbers below at times.

2. Please indicate the first line of a new paragraph either by indenting (the universal standard), or if you wish, provide spaces between paragraphs. These is a basic rule of writing, albeit violated by Microsoft Word Normal style. When in doubt consult the Chicago Manual of Style. Otherwise, your reader cannot tell where the next paragraph begins. Especially see what a problem this is in your References section!

The fourth paragraph of the introduction summarizes the paper in extensive detail. I suggest you trim this paragraph down substantially, present the data, and then tell summarize your findings.

What population of cells in the gastric antrum of their mice express Axin2CreErt2?

Fig 1D versus Fig 2D - why is there a log difference in CFU in the +/+ mice? Does this affect the interpretation of the results?

Results with the Lgr4/5 deleted mice are confirmed with RSPO3 deleted mice. It is puzzling that all of the P values are borderline here, though. Perhaps due to low numbers of mice?

Fig 5B figure legend is confusing. It says immunofluorescence labeling for GSII and GIF...but it shows qRT-PCR for Lgr4 and Lgr5.

Please show standard deviations and not standard errors. From the "Guidelines for reporting statistics in journals published by the American Physiological Society: the sequel" doi:10.1152/advan.00022.2007
"Guideline 5. Report variability using a standard deviation: The distinction between standard deviation and standard error of the mean is far more than cosmetic: it is an essential one. These statistics estimate different things: a standard deviation estimates the variability among individual observations in a sample, but a standard error of the mean estimates the theoretical variability among sample means..... To summarize, in most experiments, it is essential that an investigator reports on the variability among the actual individual measurements. A sample standard deviation does this: it describes the variability among the actual

experimental measurements."

The Pfannkuch (2018) citation is to a bioRxIV paper. The Pfannkuch paper was published in FASEB J in 2019. N.b. I don't usually check references but I was interested so I looked for the paper. So then I looked at Zimmerman (2017).

Page 3: The statement "ADP heptose has been shown to act as a central mediator of H. pylori-driven inflammation by inducing NF-κB signaling via alpha kinase 1 (ALPK1) and TRAF-interacting protein with forkhead-associated domain (TIFA) (Zimmermann et al, 2017)" is incorrect. Zimmerman identified a different mediator, HBP. I can see how you got there, but maybe you can reword to be more accurate.

Plunger plots in every figure should be replaced with scatter plots. Plunger plots lack important information that scatter plots contain, and that allow evaluation of the distribution of the data. c.f. Weissgerber et al. (2015) Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. PLoS Biol 13(4): e1002128. doi:10.1371/journal. pbio.1002128

Referee #3:

The manuscript by Wizenty et al investigates the role of components within the Rspo-Lgr signaling axis in response to H. Pylori infection in the antrum region of the mouse stomach. Using an impressive array of genetic mouse models, the authors suggest that Lgr4 and not Lgr5 is the primary receptor for Rspo3 mediating the gland hyperplastic response to H. Pylori infection. The authors furthermore indicate that the Rspo-Lgr axis primes antrum gland base cells for initiating an inflammatory response by upregulating components of the NF-KB signaling cascade. The suggestion that Lgr4 and not Lgr5 is mediating the hyperplastic response to Rspo3 upon infection is novel, and the potential link to innate epithelial immunity certainly exciting. However, before solid conclusions regarding the role of specific components within the putative Rspo-Lgr-NF-KB signaling axis can be drawn, several concerns will need to be addressed:
Major:
1. As explicitly stated by the authors, the loss of Lgr4 in Axin2CreERT2;Lgr4fl/fl animals induces a dramatic loss of Lgr5 expression in addition to the expected loss of Lgr4. Given that a key question addressed by the authors is the role of specific Rspo-receptors in the hyperplastic response to H. Pylori infection, this issue will need to be further investigated. Does the loss of Lgr4 lead to subsequent loss of gland base Lgr5+ cells which has previously been demonstrated to play protective roles in the response to H. Pylori infection, which might then be the true functional consequence of Lgr4 loss? Judging from the current results, the phenotype observed in the Lgr4fl/fl animals could well be the result of the combined loss of both Lgr4 and Lgr5, and hence no solid conclusions can be drawn on the unique role of Lgr4 in the response to H. Pylori infection. Therefore, an extensive investigation of how Lgr4 loss also induces loss of Lgr5 will need to be performed. Furthermore, in addition to the qPCR data displayed to demonstrate the loss of Lgr upon knockout, it would add value to display IHC/ISH data to clearly demonstrate that these receptors are lost uniformly in the tissue and not in specific subsets of cells or in specific locations within the tissue.
2. The authors quantify the extend of H. Pylori colonization in the various genetic mouse models by isolation and subsequent culture of H. Pylori to estimate the CFU per gram of tissue. However, in the control animals, the CFU values varies dramatically between Lgr4 and Lgr5 controls. Can the authors rule out confounding factors that affect the extend of colonization in the various setups to ensure that animals are indeed infected to the same extend? Furthermore, it is essential to demonstrate that H. Pylori does indeed colonize the base of glands across models to ensure a uniform experimental setup allowing conclusions to be drawn on the various genetic backgrounds utilized.
3. The authors demonstrate that loss of Lgr4 causes a reduction in the supposedly protective gland base hyperplasia response and hence gives rise to a higher extent of colonization in the glands. However, from the microarray data the authors find a lower expression of innate inflammatory markers. As it seems that the array data is performed on whole antrum tissue, it is surprising that the increased colonization in glands does then not subsequently lead to a higher influx of immune cells in the lamina propria to counteract the suggested abrogated epithelial response to gland colonization. Given that the authors from the title of the manuscript claim that antrum stem cells promote inflammation, which per definition involves more than just the epithelial tissue component, the authors should address what functional consequences this increased H. Pylori colonization in Lgr4fl/fl animals have for immune infiltration and inflammatory responses to H. Pylori infection, and whether this eventually causes epithelial destruction and ulceration.

Minor:
1. In figure 1, 2 and 3 the authors provide IHC images to demonstrate the expression of various markers in control infected versus infected floxed infected animals. It would strengthen the interpretation of the consequences of the genetic perturbations if the authors could provide images in parallel of uninfected control tissue as reference.
2. The authors provide Ki67 stainings to demonstrate the hyperplasic response to H. Pylori infection in the various genetic mouse models utilized. The number of Ki67+ cells will however need to be quantified and normalized to total cell numbers across experimental setups in infected controls, infected experimental and non-infected controls before the effect of H. Pylori infection on tissue hyperplasia can be appreciated.
3. Along similar lines, the expression level of p65 in figure 3G will need to be quantified and normalized to DAPI or similar in non-infected controls, infected controls and infected experimental animals to appreciate the effect of genetic perturbations and infection on p65 expression levels. Supporting data in the form of western blotting or similar would further strengthen findings

from IHC quantifications.
4. In figure 4 the authors use qPCR to investigate the role of ADP-heptose and supposed NF-KB signaling activation in epithelial organoids in various medium compositions. The interpretation of the effect of ADP-heptose treatment in the various medias is however difficult as all displayed data is normalized to full medium control. Given that the expression level of a number of targets in various media is displayed in figure 5A, it could help data interpretation if the data in figure 4C and D was normalized to the non-ADP-heptose/TNF-alpha treated controls within individual medias.
5. In addition to the above, it would strengthen the evidence of ADP-heptose signaling functioning via NF-KB to display IF images of p65 with supposed nuclear localization in FM + ADP-heptose versus FM controls.
6. The authors utilize a genetic mouse model with repressed NF-KB function to investigate the role of the signaling pathway in differentiation of secretory cells with protective roles during H. Pylori infection. However, it is not clear to this reviewer how this conditional superrepressor model was implemented exactly. Which Cre-driver was used to activate expression of the superrepressor?
7. The authors hypothesize that a conserved response in gland/crypt bases throughout the gastrointestinal tract might have evolved to sense microbial colonization in this niche. It could certainly be interesting to follow up this notion by investigating whether systemic exogenous Rspo delivery could elicit such a conserved response in the form of p65 upregulation across glands in the GI tract.

Given that these above major and minor concerns can be addressed to justify the conclusions drawn from the presented data, the findings and conclusions could be of interest for the readership of The EMBO Journal.

We would like to thank the reviewers for their valuable comments that we address below in the point-by-point response:

Referee #1:

The manuscript by Wizenty J et al, entitled "Antrum stem cells promote inflammation and gland remodeling upon exposures to H.pylori", describes some interesting and useful observations regarding the role of Lgr4+ and Lgr5+ antral crypt progenitors in the response of the gastric mucosa to H. pylori infection. In previous work from this lab, Dr. Sigal first described the role of stromal R-spo3 signaling on aAxin+Lgr5- antral stem cells during homeostasis and H. pylori infection (Sigal M et al, Nature 2017, and then went on to show that upregulation of Rspo3 by H. pylori infection leads to secretory differentiation of Lgr5+ cells which contribute to host defense (Sigal M et al, Nat Cell Biol 2019). In the current submission, the authors analyze in more depth the involvement of Lgr4 and Lgr5 receptors in regulating gland remodeling upon H. pylori infection, with a novel link shown between Rspo and NF-kB signaling. Overall, the data are of interest and provide convincing evidences on the requirement of Lgr4 receptor, but not Lgr5, in directing the H. pylori response in the gastric antrum. Given the limited data to date on Lgr4+ cells (in contrast to voluminous reports on Lgr5+ cells), this report fills an important gap and will be valuable to the field. However, the study nevertheless has a number of weaknesses and limitations, as outlined below, and I do have some concerns in regards to the interpretation of the results and conclusions drawn.

> *We thank the reviewer for their careful consideration of our manuscript and the constructive comments that helped us to improve our manuscript experimentally.*

Major concerns:
1. Introduction of Lgr4. Perhaps more rationale should be provided in the Introduction regarding why Lgr4 is studied here. In fact, the two previous papers pointed to the Axin2+Lgr5- cells in the gastric isthmus as mediating responses to Rspo3. And the one in situ hybridization figure from the 2017 paper (Extended Data Fig. 8d) did show expression of Lgr4, but not Lgr5 or Lgr6, in this isthmal region. However, it is likely a bit misleading to state that "Lgr4 is expressed throughout the gland" as sort of implies that Lgr4 is expressed ubiquitously in every antral epithelial cells, which is probably not the case. Has Lgr4 expression been further studied in single cell studies or CreERT mapping or any other technique. Perhaps better to say that Lgr4 is more broadly expressed above the antral gland base with a concentration in the antral isthmus region.

> *We have now introduced the rationale for this paper, stating explicitly that our previous work showed responses of Axin2+/Lgr5+ as well as Axin2+/Lgr5- cells to R-spondin. We also performed single molecule RNA in situ analysis and found Lgr4 to be expressed broadly throughout the gland with concentration in the antral isthmus region (Figure EV1A, B). By analyzing scRNA-seq data we confirmed a broadly expression of Lgr4 within the gland. (Figure EV1C).*
>
> *Ll. 72-75 "We have previously shown that Rspo3 drives expansion of rapidly cycling Axin2+/Lgr4+/Lgr5- cells in the isthmus, as well as slowly cycling Axin2+/Lgr4+Lgr5+ basal cells differentiate into secretory cells leading to hyperplasia in the antrum (Sigal et al., 2017; Sigal et al, 2019; Sigal et al., 2015). While this suggests that both Lgr4 and Lgr5 are likely to play a role in mediating the effects of Rspo, their distinct roles in driving antral pathobiology remain elusive."*
>
> *Ll. 91-96 "Lgr5 and Lgr4 are the Rspo receptors expressed in gastric epithelial cells. While Lgr5 itself is a Wnt target gene, Lgr4 is regulated differently (Barker et al, 2007). Expression of Lgr5 is thus limited to base cells, where Wnt/ Rspo concentrations are highest, while Lgr4 is broadly expressed throughout the antral gland. In-situ hybridization and analysis of publicly available single cell RNA-seq data confirmed the broader expression of Lgr4 in the stomach and we noticed the highest concentration of Lgr4 in the isthmus (Fig EV1A, B, C)."*

2. Characterization of conditional knockout of Lgr4 and Lgr5. The analysis and immunofluorescent staining of antral glands from the Axin2-CreERT conditional knockout mice is shown in Supplementary Figure 1. However, while it is stated correctly that there were "no major pathologies",

the analysis is a bit unsatisfying as the reason for the particular stains is not well explained and the images a bit hard to appreciate. Perhaps it should be clarified once again from the Sigal et al 2019 study that the basal antral cells are secretory mucous cells that are derived from (and may overlap with) basal Lgr5+ cells, and that they differentiate in an Rspo3 dependent manner. It was previously shown I think that lectin binding with GSII and expression of GIF overlap a bit with the Lgr5+ cells at the base (See Sigal et al 2019, Fig. 1c, Fig 2e, f). In the gastric corpus, GSII+ cells are distinct from GIF+ cells, although a subset of secretory progenitors are positive for both (i.e. rare GSII+GIF+ cells). Are there in fact double positive GSI++GIF+ cells in the antrum?

In the antrum, secretory cells in the base are GSII+ and GIF+ (double positive), which we visualize by co-staining for GSII and GIF, however there are more GSII+ cells compared to GIF+ cells (Figure EV2B). Therefore, we separately quantified GSII and GIF normalized to control mice in all mouse models, demonstrating that both markers, predominantly upon infection, are regulated by Lgr4, but not Lgr5 (Figure 1 and 2).

Ll. 109-111 "To visualize mucous gland base cells in the antrum we used immunofluorescence labelling for lectin GSII (GSII) and gastric intrinsic factor (GIF). Only a subpopulation of GSII+ cells were GIF positive (Fig 1A, Fig EV2B)."

3. With conditional knockouts, the authors suggest no change at baseline in "distribution" of GSII+ and GIF+ cells in the antrum, but their numbers appear a bit lower with knockout of Lgr4 and Lgr5. Quantification of "GSI+ area" shows a trend, but GIF staining is not quantified needs to be. PCR suggests that gene expression of GIF is decreased in both cases. Other genes that could be studied by PCR include TFF2 and MUC6 (which is measured in Fig. 1). What about expression of pit cell markers such as MUC5AC? Ki67 is stained for and appears lower in Lgr4F/F mice but is not quantified, and should be done in all four lines. Further, only one time point (day 14) is examined - was this phenotype stable with longer followup? At say 1-3 months? Given the requirement of Rspo signaling for progenitor cell function, it would be surprising to show that loss of Rspo receptors had no longer term consequences. Can antral 3D spheroids be grown from Lgr5fl/fl or Lgr4flf/fl mice? Finally, the most notable finding is that conditional knockout of Lgr4 leads to marked downregulation of Lgr5 expression. This needs perhaps a bit more explanation.

For Figure 1 (data on Lgr5) and 2 (data on Lgr4) we have quantified GSII, GIF, Ki67 and Muc5ac in uninfected and infected Lgr4 KO mice, Lgr5 KO mice and littermate controls. Both figures together now provide data to all four lines . We also added more samples for qPCR analysis. In Lgr5 KO mice we observed no significant reduction in base cell markers by qPCR and immunofluorescence in the uninfected and the infection condition. In Lgr4 KO mice there was a significant reduction in expression of Lgr5, Axin2, Gif and a trend for Pgc, Tff2 and Muc6, while immunofluorescence for GSII indeed showed a trend towards reduction of base cells, which we not state more explicitly in the text. Upon infection, we observed an expansion of the gland base and proliferative compartment in WT and Lgr5 KO mice, but not in Lgr4 KO mice. Thus, we conclude that Lgr4 expression is required to mediate epithelial gland base responses to infection, while depletion of Lgr5 alone does not alter the gland base.

The (partial) KO we achieve in our mouse model, is relatively similar at d14 (uninfected) and after 2-month of infection. Our previous data using Rspo3 KO mice also showed that changes seen upon depletion are not different from 2 weeks and 2 months, therefore we believe that depletion of Lgr4 in uninfected mice will not provide new fundamental insights. We now point out that we do see differences also in the homeostatic conditions (such as loss of GSII+ cells), but that the phenotype is rather mild and more obvious upon infection.
In fact, the system appears to be not fully dependent on Rspo-Lgr signaling at steady state, while responses to injury and infection require this signaling pathway. This is also consistent to our findings in the colon where Rspo3 is essential for epithelial recovery, while loss of Rspo3 impaired crypt regeneration upon injury (Harnack et al., 2019).

We found that removal of the Lgr ligand Rspo from organoid cultures led to a strong reduction of stemness and inflammation (Figure 6), therefore instead of depleting the receptors, we applied the different media conditions to explore in effects of Rspo-Lgr signaling in vitro.

The fact that Lgr5 itself is a Wnt target gene explains why Lgr4 KO causes reduced Wnt signaling activity (evidenced by reduced Axin2 and Sox9 expression, as shown in Figure 2E, F and 3A, B, C) and reduces Lgr5 expression, which is a Wnt target gene. We now explain this in more detail in the text:

Ll. 91-92 "While Lgr5 itself is a Wnt target gene, Lgr4 is regulated differently (Barker et al, 2007)."

Ll. 131-133 "A significant reduction in expression of Lgr5, Axin2 and Gif was detected in uninfected Lgr4$^{-/-}$ mice compared to uninfected control littermates (Fig 2E), which indicated that Lgr4 is required for Lgr5 expression and Lgr5+ cell differentiation."

Ll.146-149"Gene set enrichment analysis (GSEA) confirmed that loss of Lgr4 (which, as shown above also leads to loss of Lgr5 expression) led to a reduction of the antral stem cell signature (Barker et al., 2010) and genes associated with beta-catenin signaling (Herbst et al, 2014) (Fig 3A, B). The downregulation of the Wnt target genes Axin2 and Sox9 was validated by qPCR (Fig 3C)."

4. H. pylori infection. The point of the study is that much greater changes are seen in the conditional Lgr knockout mice after infection with H. pylori, given the normal response requires Rspo3 signaling. However, there are issues interpreting this data in that all the appropriate controls are not included. Thus, to interpret this data, we need to see both the Lgr5fl/fl infected and uninfected mice along with the corresponding infected and uninfected WT (i.e. Lgr5+/+) mice. There are in fact several comparisons that need to be made and we are only shown a few of them. Thus, there appears to be an expansion of GSII+ mucous cells in both the Lgr5+/+ and Fl/FL mice which is interesting, as this is seen in the corpus and has been called SPEM by some. GIF+ cells appear to be decreased, although they are not quantified (and should be). Finally, proliferation needs to be quantified. Most interesting is that there is no change in CFU with Lgr5 deletion, even though the previous study indicated that knockout of Rspo3 or Lgr5+ cells led to increased H. pylori colonization. Nevertheless, the overall conclusion seems right, which is that there is a more minimal effect with conditional Lgr5 gene deletion.

We now provide comprehensive data (uninfected and infected) on Lgr5 KO (Figure 1) and Lgr4 KO mice (Figure 2) together with the respective control mice. Quantifications for GSII, Gif, Ki67 and Muc5ac have been added. We agree with the reviewer that it is interesting that Lgr5 cell loss leads to increased colonization with H. pylori (previous publication Sigal et al. 2019), while as seen here mere loss of Lgr5 expression does not. We believe this is because Lgr5 expression is not necessary to maintain the gland base secretory cells, while Lgr4 expression is important. We have now highlighted this issue in the text:

Ll. 122-125 "Although in an earlier study we showed that selective deletion of Lgr5-expressing cells leads to increased colonization with H. pylori (Sigal et al., 2019), the current data suggests that this phenomenon as well as the epithelial response to infection does not require Lgr5 expression."

5. In contrast, more major changes were seen with conditional deletion of Lgr4 under conditions of H. pylori infection. There appears to be a significant loss of GSII+ cells and a loss of GIF+ cells. However, interpreting this is a bit tricky since data from uninfected Lgr4fl/fl is not shown. Most likely this reflects a lack of expansion of the GSII or GIF lineages, but in any case, the area of these lineages needs to be quantified, as does Ki67 staining which is likely decreased. Finally, the major finding is apparently the change in colonization by H. pylori. Please introduce this as "H. pylori CFU" as CFU more often in Lgr5 papers refers to stem cell quantitation via growth in soft agar. In any case, conditional knockout of Lgr4 appears to lead to a marked increase in H. pylori colonization. However, it is odd that the control here has a different level of colonization than the control in Figure 1. In addition, if one "combines" the two figures, it is clear that there is no significant difference between Lgr5fl/fl and Lgr4fl/fl mice in their colonization, both being around 8 x 104. Some comment is needed - perhaps in the methods indicating the two groups were inoculated differently and that there is variability from experiment to experiment. In any case, given findings in Sigal et al 2019, this is hard to interpret as in that study, Lgr5 cells gave rise to the secretory lineage, but here Lgr4 cells are the origin of "gland base expansion." What is happening to Lgr5 cells here. The measure of Lgr5 gene expression is

confusing, as the only comparison is Lgr4+/+ infection and Lgr4fl/fl infected and data presented as fold change with Lgr4+/+ infected included as the "control." In Supplementary Figure 1e, it was shown that at baseline, Lgr5 was expressed at much lower levels in uninfected Lgr4fl/fl mice compared to Lgr4+/+ mice. So is Lgr5 upregulated with H. pylori infection in Lgr4fl/fl mice, or is Lgr5 more downregulated in infected Lgr4+/+ mice? In the end, we need to see data from all 4 groups of mice to interpret the findings here.

As described above, we have now provided data for all four mouse lines as well as the necessary quantifications (Figure 1-3). The term H. pylori CFUs has been added. The reviewer is indeed correct in assuming that infection in different experiments with independent H. pylori cultures leads to some variability from experiment to experiment. From our experience, CFUs are most comparable for one "round" of infection.
Indeed, we believe that Lgr4 is important for expansion of gland base cells. Although these cells are Lgr5+, Lgr5 expression is not required for this phenotype, while Lgr4 is essential. Regarding the qPCR data on epithelial markers such as Lgr5, there was some variance in raw gene expression values in sets of uninfected vs. infected mice, leading to distorted fold-changes. We therefore decided to prepare the qPCR data for uninfected and infected conditions separately (Figure 1E, F and Figure 2E, F). An upregulation of Lgr5 in WT mice upon infection was described previously (Sigal et al., Nature Cell Biology, Figure 5) and we refer to this publication and the respective finding in the text: L. 109 "We earlier described that infection also leads to upregulation of Lgr5 (Sigal et al., 2019)."

6. Link between Rspo3 signaling and NF-kB pathways. Using whole transcriptome microarray, the authors observed a significant downregulation of multiple genes related to the NF-kB signaling in infected Lgr4fl/fl relative to WT controls. Using Myh11CreERT/Rspo3fl/fl mice, as well as in vitro antral organoids, they further analyze the relationship between R-spo3 and NF-kB signaling activation and conclude that Rspo3-Lgr4 axis is required to drive inflammatory response to H. Pylori. While the association between Rspo3-Lgr4 and NF-kB signaling appears evident, the proposed direct connection as well as the specificity of the cells involved is not strongly convincing or sufficiently fleshed out. First, while some NF-kB target genes are shown, the authors suggest that "most of the top downregulated genes were linked to innate immunity." How many genes were downregulated and how many were linked to innate immunity- presumably this was more than half and hopefully closer to two third. Perhaps a supplementary table with all of the genes listed and scored could be included. Second, through IHC of p65, the author suggested that NF-kB signaling activation to be restricted to the stem cell compartment under normal conditions. Immunofluorescence analysis for p65 (Fig. 6A) appears to show stronger signal intensity at the level of the isthmus, with broader labelling of the gland base can be observed in infected mice (Fig. 3G), but in both cases p65 seems to be absent from the most basal cells, which are typically occupied by the basal secretory cells. Thus, this data raises a number of questions that are not addressed. First, are the NF-kB regulated cytokines (Cxcl10, Ccl2, Cxcl1 and Cxcl2) expressed and secreted by antral epithelial cells? We as yet have no evidence for this, as the microarray analysis was performed on whole antral tissue. Second, if they are expressed by antral epithelial cells (and not the typical immune cells), are they expressed by the p65+ antral isthmus progenitors or by the GSII+ and GIF+ antral secretory cells? The authors suggest that NF-kB signaling is reduced in Lgr4fl/fl mice but no p65 staining is shown in these mice (on in the Rspo4fl/fl mice). Thus, where are Cxcl10, Ccl2, Cxcl1 and Cxcl2 produced?

We thank the referee was these helpful suggestions and insightful questions. We have now performed several experiments to further characterize the Rspo-NF-kB connection:

Table EV1 lists all significant downregulated genes (fold change > 1,5) from the microarray and highlights 63 of 157 genes related to inflammatory response via NF-kB. To further visualize this, we used DAVID 6.7 and Revigo and mapped GO terms pertaining to Lgr4 versus Lgr4 via NFKB (Figure EV3). This analysis indicates that NF-kB target genes downregulated in Lgr4$^{-/-}$ mice fall into the categories of immune response and neutrophil infiltration.

To differentiate between total levels of p65 and also activated p65 we have now additionally used p65 K310 antibody for staining. Our new data shows that p65 and activated p65 K310 are present predominantly in the Ki67+ isthmus and (less) in gland base secretory cells with downregulation upon Lgr4 KO in vivo (Figure 3E, F, G). Using a NF-kB reporter mouse line

(k-EGFP) we localized active NF-kB in proliferative cells and in a subset of GSII+ cells in organoids (Figure EV5).

We show by microarray analysis and qPCR (on organoids comprised exclusively of epithelium) that cytokines Cxcl1, Cxcl2, Cxcl10, Ccl2 are expressed by antral epithelial cells. However, to show localization of the protein, we additionally stained sections with an anti-Cxcl1 antibody. Like p65, Cxcl1 is present predominantly in the isthmus in antrum of infected mice, but not in Lgr4 KO (Figure 3I).

In summary we used four separate methods (nuclear p65, posttranslational modified p65, indicative of activation, NF-kB target genes, and k-EGFP reporter mice and organoids) to demonstrate both activation and localization of p65 in infected and uninfected antral epithelium.

7. 3D organoid experiments. Fig. 4 and 5. This presentation is more than a bit confusing. The author notes that organoids grown in full media (FM) have a baseline tone of NF-kB signaling higher than Wnt/Rspo free medium, based mainly on PCR measurements of cytokine genes. They then want to address the question as to why this does not seem to be the case in vivo upon single KO for Lgr5 or Lgr4. However, to start with, it is not clear that the authors have rigorously examined NF-kB signaling in the single knockout animals. In any case, the authors then proposes that baseline function can be compensated when only one receptor is present. Therefore, they go on generate double (Lgr4flf/fl;Lgr5fl/fl) knockout mice, which have reduced GSII+ area, and reduced GIF and PGC gene expression. In addition, they show significant downregulation of NF-kB genes. However, it is not clear that any of these findings represent significant differences from single knockout (e.g. Lgr4fl/fl) mice, which I would argue are nearly identical. Indeed, while the authors may want to raise the notion of compensatory effects by Lgr5 in the Lgr4fl/fl mice, in fact Lgr5 gene expression in Lgr4fl/fl mice is more than 80% decreased (Fig. S1E) and probably not much different than Lgr5 expression in the double knockout animals. However, apart from the actual level of expression, does Lgr5 expression change in location or expand beyond the crypt base in Lgr4fl/fl mice?

We have now performed the respective quantification that indeed confirmed what the reviewer observed (Figure 2 A, B, C). To avoid confusion we therefore removed the double KO mice, as it does not provide any additional insight, and instead performed additional experiments and analyses on Lgr4$^{-/-}$ mice to determine the role Lgr signaling for NF-kB (Figure 3).
We have now also performed in situ hybridization in Lgr4$^{-/-}$ for Lgr5 and did not find an expansion beyond the crypt base (Figure EV1A).

8. Inhibition of NF-kB signaling. Fig. 6. This is a useful experiment, with a dominant negative IkB, but this is not presented or explained well. Is this a constitutive IkB mouse, with inhibition in every cell type? It would be helpful to clarify just a bit more the features of this mouse. The finding of reduced glandular differentiation in vitro and in vivo, along with increased proliferation, is interesting and consistent, but still a bit unclear where this is actually occurring. The term "stem cell differentiation" is used here, but is this in the isthmus or more towards the gland base. What are the proliferating cells - are they Lgr4+? The data seem to support Lgr4+ cells as the relevant progenitors but then where do Lgr5+ cells fit in, since they appear to give rise to secretory cells in the previous paper (Sigal et al, 2019)? A bit more explanation would be helpful here. Perhaps a sentence or two could be added to the Discussion explaining the distinct roles of the Rspo3-Lgr4 and Rspo3-Lgr5 axis.

We have now provided a more detailed explanation of the features of the dominant negative IkB mouse in the text:

Ll. 257-261 “Therefore, we obtained mice with ubiquitously suppressed NF-κB activity, by using a transdominant negative mutant of IkBa (IkBaΔN) that was inserted in-frame into the beta-catenin locus (cI kappa B alpha Delta N, hereafter referred to as ΔN). Importantly, the beta-catenin protein expression and activity in these mice is not impaired (Schmidt-Ullrich et al, 2001).”

Indeed, we do believe that Lgr4+ isthmus cells can give rise to secretory cells in the gland base. Our 2019 paper shows that these cells express Lgr5, but again this does not mean that

they derive from Lgr5+ cells, instead it is likely that Lgr4+ cells can give rise to secretory gland base cells upon exposure to R-spondin that then express Lgr5. We now discuss this as we believe that the R-spondin-Lgr4 axis is highly relevant for epithelial responses, while Lgr5 expression is a marker of R-spondin signaling but largely dispensable for R-spondin effects:

Ll. 293-297 "Our data here demonstrate that Lgr4 represents a critical receptor for mucosal responses to H. pylori in the antrum. Lgr4 also controls Lgr5 expression, however Lgr5 alone does not impact H. pylori infection, indicating that while Lgr5+ cells are critical in the context of infection, their maintenance and function is not driven by Lgr5 alone, but requires Lgr4 expression. Therefore, the Rspo-Lgr4 axis is highly relevant for epithelial responses, while Lgr5 expression is a marker of Rspo signaling, but is largely dispensable for Rspo effects."

By visualizing the gland base upon suppression of NF-kB in vivo, we show high levels of proliferation but a lack of the secretory cells normally present at this location, which is in line with the gene expression data. Our data therefore suggest that activation of NF-kB in the isthmus is important for differentiation of proliferative cells into secretory gland base cells.

Minor concerns:
-The authors refer to the 129;129P2-ctnnb1tm(NFKBIAΔN)1Rsu mice as NF-kB-/-. While is understandable this choice the nomenclature used may be confusing and therefore a different nomenclature is recommended.

This has been changed to ΔN, the standard short form nomenclature used for this mouse in publications.

- The authors refer to "inflammation" frequently, but never really show (even in H. pylori-infected mice) an increase in leukocytes which is the definition of inflammation. Perhaps they should explain early on that by inflammatory responses, they mean the increase in proinflammatory cytokines.

We now provide new experimental data on immune cell infiltration (neutrophils, macrophages, T cells) in all 4 lines of Lgr4 KO as well as Lgr5 KO mice (Figure 5, Figure EV4). Using immunofluorescence, we revealed that, Lgr4, but not Lgr5 alone, is required for neutrophil infiltration into the mucosa, thereby demonstrating a functional consequence of altered epithelial cytokine expression on immune cell responses. In main text we change wording to differentiate between inflammation (meaning involvement of the immune cell infiltrates) versus increase in pro-inflammatory cytokines (in this case by the epithelium)

e.g. Ll. 168-170 "Taken together, our in vivo experiments reveal that Lgr4 is required for NF-κB activation and expression of pro-inflammatory chemokines in response to H. pylori infection."

-The authors show that constitutive inhibition of NF-kB signaling abrogates secretory cell specification in the mouse antrum. Is this effect specific of the mouse antrum or can be generalized to the gastric mucosa? What about the small intestine?

We thank the reviewer for this comment. Indeed, NF-kB has been studied recently in the small intestine. It has been shown that NF-kB is required for differentiation of progenitors into secretory Paneth cells in the small intestine. A reference to this study is now included in the text.

Ll. 341-343 "In the small intestine, NF-κB is required for proper secretory Paneth cell differentiation and epithelial homeostasis by regulating Wnt signaling and Sox9 expression downstream of NF-κB (Brischetto et al., 2021)."

-While impeding secretory cell differentiation, NF-kB inhibition does not inhibit proliferation of isthmus progenitor cells and in fact accelerates it. Has the author tested if H. pylori infections can induce crypt hyperplasia in the mouse antrum independently of NF-kB activation?

This is an interesting question and would be important to address in future studies. Here unfortunately, we were not able to infect ΔN mice.

-NF-kB inhibition strongly reduces Lgr5 expression in the mouse antral organoids (Fig. 6C). Is this true in vivo? Is Lgr4 expression altered in organoids or in this mice?

In vivo we observed no significant reduction of Lgr5, probably due to variance within the control animals. Analysis of Lgr4 expression in organoids (Figure 8B) did show upregulation, pointing towards a compensatory mechanism upon suppression of NF-κB.

- In the discussion, the authors describe evidence that Rspo signaling drives expression of its receptor Lgr5, but no reference is provided. Please provide a reference to support this statement. Studies from Kuo CJ et al have shown in vivo increases in Lgr5-EGFP+ cells with Rspo treatment, but I'm not sure this is the same thing as receptor upregulation. In the same paragraph, I'm not sure what "constitutive expression of Lgr4" means.

Studies by the Kuo group indeed showed an increase of Lgr5+ cells, but also of Lgr5 gene expression upon Rspo treatment. We now cite this paper. We did not want to speculate about receptor upregulation and clearly state Lgr5 expression now. Regarding the expression of Lgr4, we have now provided RNA in situ data that show a broad expression of Lgr4 in the epithelium. The term has been revised accordingly.

Ll. 283-286 "Thus, Rspo signaling itself drives expression of its own receptor Lgr5, implying that a positive autocrine feedback loop that stabilizes Wnt/Rspo signaling could exist (Yan et al, 2017). In contrast, unlike Lgr5, Lgr4 is not a Wnt target gene and broadly expressed in the epithelium of the gastrointestinal tract, including the stomach (Harnack et al., 2019; Sigal et al., 2017)."

- Subpopulation of H. pylori bacteria ... colonize the gastric glands. I'm not sure what is meant here. Do the authors mean that the bacteria colonizing the deep gastric glands are somehow different from the ones that colonize the pits? Or do they simply mean that a random subset of H. pylori bacteria somehow end up colonizing the gastric gland base. A few published studies have suggested that H. pylori can come into contact with antral stem cells. Perhaps these studies could be referenced.

WT bacteria use a specific adhesion and motility system to colonize in gastric glands. While most bacteria are free swimming, some also (as far as we are aware randomly) colonize deep in gastric glands in contact with Lgr5+ cells. We described this previously (Sigal et al., 2015). The analysis of bacterial subpopulations or virulence factors in this context appears beyond the scope for this study, however, we now reference the respective studies.

Ll. 43-45 "In addition, a subpopulation of H. pylori can form colonies deep inside the gastric glands, where stem and progenitor cells reside (Fung et al, 2019; Sigal et al, 2015)."

-In the figures' legend (Figure 6) the author describe panel G and H. These panels are otherwise not referenced in the test and not present in the panel.

We have now adjusted the text accordingly.

Referee #2:

Previously the senior author has shown that RSPO3 is important in the response to helicobacter infection in the mouse stomach. Here his group addresses which RSPO3 receptor, LGR4 or LGR5, is important. Lgr5 knockout with an Axin2 Cre has no obvious phenotype, while the Lgr4 ko fails to react to helicobacter infection. This implicates Lgr4, but there is a major problem with this interpretation noted below.

Looking at the consequences of Lgr4 knockout, they find these the Lgr4 ex/ex mice have apparently downregulated NF-κB signaling, a finding replicated by loss of the ligand RSPO3. This bit is strong and interesting but mechanistically remains a black box. In organoids they find that Wnt/RSPO

signaling makes organoids permissive for expression of putative NF-κB targets using an interesting metabolite ADP-heptose and TNFa. Fig 5 detours a bit to show that gland base cells are altered in a formal Lgr4/5 double knockout, and show by microarray/GSEA that there is decrease in TNFa genes. Building a case that NF-κB is important in the stomach, they re-analyse a mouse with globally inhibited NF-κB and find alterations in a handful of gland base markers.

I found the flow of this manuscript hard to follow, and it took me several read-throughs to get the gist. I think this is because the strength of any specific conclusion is not very strong, as evidenced by the multiple instances of n=3 experiments and p values ~0.05. In addition, they tend to show many things by one experiment, e.g. a microarray and GSEA, and don't go deeper with confirmatory experiments. They go a long way on these modestly powered experiments, and there are multiple claims not supported by experiments. So while it's likely to be mostly correct and reproducible, the level of rigor is not high.

The biggest issue with the manuscript: The Lgr4fl/fl/Axin2CreErt2 mice after tamoxifen have greater loss of Lgr5 than Lgr4 mRNA. Suppl Fig 1E validates the knockout efficacy and introduces this issue, which is confirmed in Fig 2C. Please explain why Lgr4 fl/fl mice have more loss of Lgr5 mRNA than of Lgr4 mRNA? What is going on? Can they confirm genotyping? Did they lose expression of Wnt target genes?

This results makes separation of the role of Lgr4 versus Lgr5 a challenge that is not addressed. - it appears that Lgr4 ko acts like a double Lgr4 Lgr5 ko. So any phenotype seen in the Lgr4 ko is not cleanly an Lgr4 ko. Thus, a comment like "Thus, we conclude that Lgr4, but not Lgr5, is the critical receptor..." should perhaps read something like "Thus, we conclude that Lgr4 either alone or in combination with Lgr5, but not Lgr5 alone, is the critical receptor ..." "confirmed that Lgr4 depletion" -> "confirmed that Lgr4 plus Lgr5 depletion" etc. "response to H. pylori infection is driven by the Rspo3-Lgr4 axis" -> response to H. pylori infection is driven by the Rspo3-Lgr4/5 axis.

> *We acknowledge the concern of the reviewers regarding the strength of the experimental data, which we have now improved by adding several conclusive new experiments.*
>
> *<u>Rigor/ mechanistic studies:</u>*
> *We performed several new experiments to confirm our findings and generate deeper insights:*
>
> *1) p65 is expressed predominantly in the Ki67+ isthmus and (to a lesser degree) in gland base secretory cells with downregulation upon Lgr4 KO in vivo (Figure 3E, F).*
> *2) Localization of Cxcl1 and the active form of p65 (K310) predominantly in isthmus cells in vivo (Figure 3G, I).*
> *3) NF-kB binding capacity in organoids depends on Rspo, as revealed by electrophoretic mobility shift assay (EMSA) (Figure 7A, B).*
> *4) Supershift analysis in organoids demonstrating that the canonical NF-κB pathway is activated by ADP heptose and repressed upon Rspo removal and that specifically the p65 subunit is responsible for this activation (Figure 7C).*
> *5) Localization of nuclear p65 in proliferative and in a subset of gland base secretory cells in organoids (Figure EV5A) as well as localization of active NF-κB in organoids using an NF-kB reporter mouse line (Figure EV5B, C).*
> *6) Beta-catenin staining and qPCR analysis of Wnt targets in infected and uninfected Lgr4 KO mice plus controls (Figure EV2C, Figure 3C).*
>
> *Where possible, biological replicates have been added.*
>
> *This additional data now provides a sound basis for our conclusions and allowed us to re-structure the manuscript to make it easier to follow.*
>
> *<u>Lgr4 vs Lgr5:</u>*
> *Indeed, our interpretation is that Lgr4 loss will lead to a loss of Wnt signaling (Figure 2E, F, Figure 3A, B, C), which in turn results in a loss of Lgr5 expression, since it is a Wnt/Rspo target gene. Using organoids, we now demonstrate that removal of Rspo from the culture leads to reduction of Lgr5 expression confirming that Lgr5 expression also depends on Wnt*

signaling in vitro (Figure 6C). The conclusions have now been modified in accordance with the reviewer's suggestion:

e.g. Ll. 137-141 "While this makes it impossible to explore the role of Lgr4 alone, our data indicates that expression of Lgr4 is essential for gastric gland hyperplasia upon infection, gland base expansion and antimicrobial defense upon H. pylori infection, while these parameters are unaffected upon loss of Lgr5 alone."

The loss of Lgr5 expression in Lgr4 KO mice unfortunately means that we are unable to explore the role of Lgr4 loss alone. Genotyping has been performed carefully, please find a gel example below (314 bp = floxed, 230pb = WT).

Figure for referees not shown

There is only one indication what the mouse background of the various strains, and that's a 129 NK-kb. Are the other mice B6 background? If so, how do they control for strain effects versus specific gene effects in B6 x 129 crosses? A full proper nomenclature (e.g. from Jax) would aid reproducibility. It's good practice to be very clear about genetic backgrounds, especially when there are multiple crosses.

ΔN mice are 129. All other mice are B6 and all experiments were preformed using isogenic littermates as controls. There were no crosses performed with B6 x 129. We have now added the full Jax Nomenclature for all mice.

Fig 3 - they assert than GSEA shows a decreased β-catenin signature, but no clear β-catenin target genes are shown in Fig 3B and the double KO mice (since the Lgr4 is effectively a double KO) don't have a proliferation phenotype. So I'm not convinced re: the β-catenin signature shown in 3A, unless this claim is supported with analysis of some specific β-catenin target genes. They could start with Axin2.

We have now complemented the data and validated the results using the Wnt target genes: Axin2 and Sox9 (Figure 3C). In addition, beta-catenin staining was performed (Figure EV2C).

Isn't it surprising that the Lgr4 knockout (with low Lgr5) has no phenotype prior to infection? Loss of Lgr4/5 affects intestinal stem cells. Does it not have an effect on stomach? Please explain the background here.

Quantification of GSII and GIF in immunofluorescence images shows that indeed Lgr4 KO has a phenotype in homeostasis conditions (Figure 2A, C). Importantly, Lgr5 KO alone has no phenotype:

Ll. 130-133 “Notably, even in the absence of infection, gland bases of Lgr4-/- mice contained fewer GSII+ cells. A significant reduction in expression of Lgr5, Axin2 and Gif was detected in uninfected Lgr4-/- mice compared to uninfected control littermates (Fig 2E), which indicated that Lgr4 is required for Lgr5 expression and Lgr5+ cell differentiation.”

The data on downregulation of NF-KB pathways is straightforward but I would like to know the status of the indicated genes in non-infected Lgr4/5 ko mice to know if this change is due to infection.

Figure 3H now provides gene expression data for the chemokines Cxcl1, Cxcl2, Cxcl10 and Ccl2, all of which are upregulated upon infection in WT mice, but not in Lgr4 KO mice. In uninfected mice, Lgr4 KO also led to some downregulation, although this was not significant, which may be due to the very low baseline expression levels for these chemokines.

Since Lgr4/5 are the primary receptors for RSPO3, the data to this point confirm the prior reports on the importance of RSPO3 signaling and makes the new connection to NF-κB signaling.

We agree, the novelty here is that loss Lgr5 expression is not sufficient to block R-spondin signaling and we have now made this explicit in the text:

Ll. 122-125 “Although in an earlier study we showed that selective deletion of Lgr5-expressing cells leads to increased colonization with H. pylori (Sigal et al., 2019), the current data suggests that this phenomenon as well as the epithelial response to infection does not require Lgr5 expression.”

Fig 4 shows that in organoids, Wnt signaling is required for NF-κB target expression. It would be useful to show in Fig 4C that the induction of target genes like Cxcl1 by ADP-heptose in this setting was indeed due to NF-κB rather than just assuming it. This could be done with small molecule inhibitors, and testing if NF-κB was indeed nuclear. The amount of data showing NF-κB itself is activated is limited to fig 3G.

This is an important point. We now confirmed that Wnt is required for NF-kB activation and target gene expression using several experimental approaches:
- via immunofluorescence and staining for nuclear p65: p65 is present predominantly in the Ki67+ isthmus and (to a lesser degree) in gland base secretory cells with downregulation upon Lgr4 KO in vivo (Figure 3E, F)
- via immunofluorescence: Localization of Cxcl1 and the active form of p65 (K310) predominantly in isthmus cells in vivo (Figure 3G, I).
-via electrophoretic mobility shift assay (EMSA): NF-kB binding capacity is dependent on Rspo in organoids (Figure 7A, B).
- via Supershift analysis in organoids: we demonstrated that the canonical NF-kB pathway is activated by ADP heptose and repressed upon Rspo removal, and that specifically the p65 subunit is responsible for this activation (Figure 7C).
- k-EGFP reporter mouse and organoids: Localization of nuclear p65 in proliferative and in a subset of gland base secretory cells in organoids (Figure EV5A) as well as localization of active NF-kB in organoids using a NF-kB reporter mouse line (EGFP) (Figure EV5B, C).

We also tested a chemical NF-kB inhibitor (BAY11-7082), however it appeared to have toxic side-effects on organoids and is therefore not included in this study.

While the authors assert on p. 6 that they "examine their ability to activate NF-κB via the ALPK1-TIFA-TRAF6 axis" the manuscript has no experimental data addressing the ALPK1-TIFA-TRAF6 axis. They should either include such experiments (a lot of work) or just mention this is one way it could happen.

We have now changed the wording and indicated that since similar results are achieved with TNF-α , the effects are not unique to ADP heptose signaling:

Ll. 231-233 “We found similar effects of growth factor withdrawal, indicating that NF-κB activation in gland base cells is not limited to ADP heptose induced signaling and that activation via the classical TNF-α pathway also depends on Rspo signaling (Fig 6F).”

(Here I'm again a bit confused - in Sigal 2019 depletion of Lgr5 expressing cells made stomachs permissive for helicobacter. How does that finding fit here?)

> *This is indeed an interesting point. The fact that depletion of Lgr5+ cells has an impact, while depletion of the Lgr5 gene alone does not, is an important finding here and we now state this more clearly:*
>
> *Ll. 122-125 "Although in an earlier study we showed that selective deletion of Lgr5-expressing cells leads to increased colonization with H. pylori (Sigal et al., 2019), the current data suggests that this phenomenon as well as the epithelial response to infection does not require Lgr5 expression."*

Fig 5 uses a genetic Lgr4/5 double knockout to show a modest depletion of gland base cells. I would expect a significant small intestine phenotype. Did that happen in their strain? They then assert a loss of tonic NF-κB signaling using only GSEA on microarray data but don't show any specific gene changes by PCR or at the protein level, nor do they show any biochemical evidence of decreased NF-κB signaling. This is a weakness.
Fig 6 could have addressed this but...

> *Following the suggestions of reviewer 1 we focused on Lg4KO mice in more detail. The quantification of GSII and GIF in Lgr4 KO mice showed a reduced number of base cells. Therefore, we believe that double KO mice do not have a significantly different phenotype and cause an unnecessary confusion, and have now removed the double KO data from the revised manuscript.*

They look at p65 protein abundance in normal stomach but not in the single or double knockouts. It's mostly in the cytoplasm, which I think means it's not signaling. They then use a mouse with presumed inactive NF-κB via constitutive expression of an active ikb (interestingly knocked into the β-catenin locus, making these mice haploinsufficient for β-catenin; Schmidt-Ullrich 2001) and find this has altered expression of Lgr5 (why?) and differentiation markers and Cxcl2 (why is Cxcl1 change in expression not significant?). This might be similar to what was observed in the Schmidt-Ullrich in other tissues. It's interesting that the phenotype is seen in Wnt-responsive tissues like hair follicles, teeth and gastric gland bases. Could this be related to the β-catenin haplo-insufficiency?

> *We thank the reviewer for this comment, and we have now supplemented our studies with experiments that look at activated NF-kB, because as the reviewer pointed out, cytoplasmic NF-kB is indeed not signaling. However, in epithelium, even post stimulation, p65 tends to be mostly cytoplasmic. To circumvent with issue, we have used p65 antibodies that correspond to transcriptionally active NF-kB (K310) (Figure 3G).*
> *Furthermore we have investigated the p65 protein in all four Lgr4 lines (Figure 3 E, F).*
>
> *As the reviewer rightfully pointed out, the knock in is indeed into the beta catenin locus, however we discussed this with the investigators that have generated this mouse and found that the integration of the NF-kB inhibitor in-frame into the beta catenin locus does not impair expression and activity of the β-catenin protein (Schmidt-Ullrich 2001). In gastric organoids from these mice, we found a downregulation of Lgr5, which can be explained by affected Wnt signaling which is described downstream of NF-kB in the intestine (Brischetto et al., 2021, Mikuda and Schmidt-Ullrich et al. 2020). Cxcl1 may not have been significantly regulated due to heterogeneity in the control organoids. We have now modified the text accordingly.*
>
> *Ll. 257-261 "Therefore, we obtained mice with ubiquitously suppressed NF-κB activity, by using a transdominant negative mutant of IkBa (IkBaΔN) that was inserted in-frame into the beta-catenin locus (cI kappa B alpha Delta N, hereafter referred to as ΔN). Importantly, the beta-catenin protein expression and activity in these mice is not impaired (Schmidt-Ullrich et al, 2001)."*
>
> *Ll. 341-343 "In the small intestine, NF-κB is required for proper secretory Paneth cell differentiation and epithelial homeostasis by regulating Wnt signaling and Sox9 expression downstream of NF-κB (Brischetto et al., 2021)."*

Other issues:
Please describe and/or reference the generation of the Lgr4 and Lgr5 floxed mice - there is no information here about where the loxP sites are, how they were generated, and how they were validated. There is only the insufficient statement "Lgr4fl/fl mice and Lgr5fl/fl mice were kindly provided by Dr. Jan Tchorz, Novartis." And given the problem with Suppl Fig 1E, how were they genotyped, and are they sure they didn't get the genotypes confused?

> *Information regarding the position of the loxP sites has been added and referenced from the original publication by the Tchorz group. We are convinced that there is no confusion here.*
>
> *Ll. 363-367 "Mouse models were designed for both genes by flanking exon1 with loxP elements facilitating Cre-mediated recombination and excision of exon1 including part of the 5'UTR. For Lgr4, Cre-mediated excision results in a deletion of 1.9 kb including Lgr4 exon1 plus 900 bp of 5'UTR. For Lgr5, Cre-mediated excision results in a deletion of 1.6 kb including Lgr5 exon1 plus 600 bp of the 5'UTR (Planas-Paz et al., 2016)."*

A bit of nomenclature confusion - Does Lgr5 fl/fl mouse refer to before or after tamoxifen? Or both? Normally, I think fl/fl refers to mouse with floxed but intact gene, and then you indicate the cre driver, and then ex/ex refers to after Cre-mediated excision if it's a conditional (e.g. CreER) excision. But that's just me. Please make it clearer. Using the fl/fl everywhere is not precise.

> *KO was achieved by floxed sites and this has now been indicated in the text. KO mice are labeled with -/- and controls are referred to as control. All mice received tamoxifen.*

Pro tips: 1. Please number your pages. Numbering your lines would be even more helpful to the reviewer. I numbered the pages in my copy (using Acrobat) starting with the title page and may refer to those numbers below at times.

> *Thank you, and sorry for the insufficient formatting. This has been implemented.*

2. Please indicate the first line of a new paragraph either by indenting (the universal standard), or if you wish, provide spaces between paragraphs. These is a basic rule of writing, albeit violated by Microsoft Word Normal style. When in doubt consult the Chicago Manual of Style. Otherwise, your reader cannot tell where the next paragraph begins. Especially see what a problem this is in your References section!

> *This has been implemented by indenting in the text and spaces in the reference section.*

The fourth paragraph of the introduction summarizes the paper in extensive detail. I suggest you trim this paragraph down substantially, present the data, and then tell summarize your findings.

> *This has been adjusted accordingly:*
>
> *Ll. 78-85 "Here, we applied conditional knockout mice that lack Lgr4 or Lgr5 to explore their involvement into antral mucosal responses to H. pylori infection. We reveal that Lgr4 expression is required to maintain expression of Lgr5 in the gland base. Depletion of Lgr4, but not Lgr5 alone, results in a diminished hyperplasia as well reduced inflammatory response and we demonstrate that the Rspo-Lgr axis does not only promote hyperplasia but is also important for the ability of gland base cells to induce epithelial pro-inflammatory NF-κB signaling upon infection. Our data reveal an explanation for the unique ability of the stem cell compartment to respond to H. pylori infection and demonstrate a novel link between epithelial stem cells and innate immunity in the stomach.*

What population of cells in the gastric antrum of their mice express Axin2CreErt2?

> *We have already shown this in a previous publication (Sigal et al. Nature 2017).*
>
> *Ll. 62-64 The antral gland base and lower isthmus contains gastric stem cells that are characterized by high Wnt signaling and expression of stem cell markers such as Axin2 and*

Lgr5 (Barker et al, 2010; Sigal et al., 2017).

Fig 1D versus Fig 2D - why is there a log difference in CFU in the +/+ mice? Does this affect the interpretation of the results?

We do not think so – the CFUs can differ between experiments and the most reliable comparison is therefore between mice that were inoculated with the same inoculum. We have now added a comment on this in the manuscript:

Ll. 392-393 "Lgr4$^{-/-}$ and Lgr5$^{-/-}$ mice plus littermate controls were infected with separate H. pylori cultures leading to variability in CFUs between two different experiments."

Results with the Lgr4/5 deleted mice are confirmed with RSPO3 deleted mice. It is puzzling that all of the P values are borderline here, though. Perhaps due to low numbers of mice?

Yes, indeed some p values are borderline since using complex in vivo systems combined with a chronic infection model inevitably results in a certain degree of variability.

Fig 5B figure legend is confusing. It says immunofluorescence labeling for GSII and GIF...but it shows qRT-PCR for Lgr4 and Lgr5.

This has now been corrected.

Please show standard deviations and not standard errors. From the "Guidelines for reporting statistics in journals published by the American Physiological Society: the sequel"
doi:10.1152/advan.00022.2007
"Guideline 5. Report variability using a standard deviation: The distinction between standard deviation and standard error of the mean is far more than cosmetic: it is an essential one. These statistics estimate different things: a standard deviation estimates the variability among individual observations in a sample, but a standard error of the mean estimates the theoretical variability among sample means..... To summarize, in most experiments, it is essential that an investigator reports on the variability among the actual individual measurements. A sample standard deviation does this: it describes the variability among the actual experimental measurements."

We have changed all graphs to SD.

The Pfannkuch (2018) citation is to a bioRxIV paper. The Pfannkuch paper was published in FASEB J in 2019. N.b. I don't usually check references but I was interested so I looked for the paper. So then I looked at Zimmerman (2017).

This citation has now been corrected.

Page 3: The statement "ADP heptose has been shown to act as a central mediator of H. pylori-driven inflammation by inducing NF-κB signaling via alpha kinase 1 (ALPK1) and TRAF-interacting protein with forkhead-associated domain (TIFA) (Zimmermann et al, 2017)" is incorrect. Zimmerman identified a different mediator, HBP. I can see how you got there, but maybe you can reword to be more accurate.

This has been reworded.

Ll. 52-56 "ADP heptose, as well as its derivative D-glycero-β-D-manno-heptose 1,7-bisphosphate (HBP), has been shown to act as a central mediator of H. pylori-driven inflammation by inducing activation of the transcription factor NF-κB via alpha kinase 1 (ALPK1) and TRAF-interacting protein with forkhead-associated domain (TIFA) (Pfannkuch et al., 2019; Zimmermann et al, 2017)."

Plunger plots in every figure should be replaced with scatter plots. Plunger plots lack important information that scatter plots contain, and that allow evaluation of the distribution of the data. c.f. Weissgerber et al. (2015) Beyond Bar and Line Graphs: Time for a New Data Presentation Paradigm. PLoS Biol 13(4): e1002128. doi:10.1371/journal. pbio.1002128

We have changed all graphs to scatter plots.

Referee #3:

The manuscript by Wizenty et al investigates the role of components within the Rspo-Lgr signaling axis in response to H. Pylori infection in the antrum region of the mouse stomach. Using an impressive array of genetic mouse models, the authors suggest that Lgr4 and not Lgr5 is the primary receptor for Rspo3 mediating the gland hyperplastic response to H. pylori infection. The authors furthermore indicate that the Rspo-Lgr axis primes antrum gland base cells for initiating an inflammatory response by upregulating components of the NF-KB signaling cascade. The suggestion that Lgr4 and not Lgr5 is mediating the hyperplastic response to Rspo3 upon infection is novel, and the potential link to innate epithelial immunity certainly exciting. However, before solid conclusions regarding the role of specific components within the putative Rspo-Lgr-NF-KB signaling axis can be drawn, several concerns will need to be addressed:

We thank the reviewer for their positive feedback and constructive comments.

Major:
1. As explicitly stated by the authors, the loss of Lgr4 in Axin2CreERT2;Lgr4fl/fl animals induces a dramatic loss of Lgr5 expression in addition to the expected loss of Lgr4. Given that a key question addressed by the authors is the role of specific Rspo-receptors in the hyperplastic response to H. Pylori infection, this issue will need to be further investigated. Does the loss of Lgr4 lead to subsequent loss of gland base Lgr5+ cells which has previously been demonstrated to play protective roles in the response to H. Pylori infection, which might then be the true functional consequence of Lgr4 loss?

Yes, this is our interpretation: Loss of Lgr4 alters Wnt signaling. We have now validated these results by performing qPCR for the key Wnt target genes: Axin2, Sox9, Lgr5 (Figure 2E, F, Figure 3C). The lack of Wnt signaling seems to render the gland incapable of expanding the gland base secretory cells.

Ll. 138-141 "While this makes it impossible to explore the role of Lgr4 alone, our data indicates that expression of Lgr4 is essential for gastric gland hyperplasia upon infection, gland base expansion and antimicrobial defense upon H. pylori infection, while these parameters are unaffected upon loss of Lgr5 alone."

Judging from the current results, the phenotype observed in the Lgr4fl/fl animals could well be the result of the combined loss of both Lgr4 and Lgr5, and hence no solid conclusions can be drawn on the unique role of Lgr4 in the response to H. Pylori infection. Therefore, an extensive investigation of how Lgr4 loss also induces loss of Lgr5 will need to be performed. Furthermore, in addition to the qPCR data displayed to demonstrate the loss of Lgr upon knockout, it would add value to display IHC/ISH data to clearly demonstrate that these receptors are lost uniformly in the tissue and not in specific subsets of cells or in specific locations within the tissue.

We believe that Lgr4 is important for induction/maintenance of R-spondin signaling in the stomach. Our data reveal that loss of Lgr5 expression is not sufficient, while loss of Lgr4 does result in a phenotype, which indeed involves the loss of expression of Lgr5, an R-spondin target gene. Indeed, this means we cannot be certain that Lgr4 alone is critical, and we have now adjusted the manuscript accordingly throughout. We mapped Lgr homologues in WT and upon KO (Figure EV1A, B) and found that Lgr4, which is broadly expressed in the epithelium in WT, is uniformly reduced in the epithelium upon Lgr4 KO. Also, in situ hybridization for Lgr5, which is restricted to base cells in WT confirms that it is reduced upon Lgr4 KO and upon Lgr5 KO.

2. The authors quantify the extend of H. Pylori colonization in the various genetic mouse models by isolation and subsequent culture of H. Pylori to estimate the CFU per gram of tissue. However, in the control animals, the CFU values varies dramatically between Lgr4 and Lgr5 controls. Can the authors rule out confounding factors that affect the extend of colonization in the various setups to ensure that

animals are indeed infected to the same extend? Furthermore, it is essential to demonstrate that H. Pylori does indeed colonize the base of glands across models to ensure a uniform experimental setup allowing conclusions to be drawn on the various genetic backgrounds utilized.

> *The CFU can vary from experiment to experiment and in our experience the comparison between littermates that were infected with the same inoculum is the most reliable measure. We have now added a comment on this issue in the Methods section. Gland colonization in the antrum is seen in every mouse infected with WT H. pylori (Figure EV2A).*
>
> *Ll. 392-393 "Lgr4$^{-/-}$ and Lgr5$^{-/-}$ mice plus littermate controls were infected with separate H. pylori cultures leading to variability in CFUs between two different experiments."*

3. The authors demonstrate that loss of Lgr4 causes a reduction in the supposedly protective gland base hyperplasia response and hence gives rise to a higher extent of colonization in the glands. However, from the microarray data the authors find a lower expression of innate inflammatory markers. As it seems that the array data is performed on whole antrum tissue, it is surprising that the increased colonization in glands does then not subsequently lead to a higher influx of immune cells in the lamina propria to counteract the suggested abrogated epithelial response to gland colonization. Given that the authors from the title of the manuscript claim that antrum stem cells promote inflammation, which per definition involves more than just the epithelial tissue component, the authors should address what functional consequences this increased H. Pylori colonization in Lgr4fl/fl animals have for immune infiltration and inflammatory responses to H. Pylori infection, and whether this eventually causes epithelial destruction and ulceration.

> *We have now assessed immune cell infiltration by immunofluorescence. Upon Lgr4 KO we observed downregulation of neutrophil infiltration (MPO), but not of macrophages (IBA1) and T cells (CD3) (Figure 5, Figure EV4). The reduction of neutrophils can be explained by the reduction of chemokines such as Cxcl1 in the knockout, which predominantly attracts neutrophils. Epithelial destruction or ulceration was not observed.*
>
> *Ll. 192-202 "We therefore asked if mucosal inflammation is dependent on Lgr4 or Lgr5. Using immunofluorescence, we stained for MPO, IBA1 and CD3 to analyze the presence of neutrophils, macrophages, and T cells, respectively, in Lgr4$^{-/-}$ mice in uninfected and infected conditions as well as in control mice. As expected, infection led to high mucosal influx of all analyzed immune cells, predominantly in the proximal antrum. Interestingly in infected Lgr4-/- mice, neutrophil infiltration was significantly diminished, indicating that a lack of proper epithelial Lgr signaling has a functional consequence with regards to recruitment of inflammatory cells (Fig 5A, B). Recruitment of macrophages and T cells was not significantly altered in these mice, which may point towards a distinct immune cell infiltration pattern with various influence factors in gastritis that warrants further investigation (Fig EV4A, B). Equivalent analysis of infected Lgr5$^{-/-}$ mice failed to detect altered immune cell infiltration (Fig 5 A, B, Fig EV4A, B)."*

Minor:
1. In figure 1, 2 and 3 the authors provide IHC images to demonstrate the expression of various markers in control infected versus infected floxed infected animals. It would strengthen the interpretation of the consequences of the genetic perturbations if the authors could provide images in parallel of uninfected control tissue as reference.

> *This has now been implemented in Figure 1-3, Figure 5, Figure EV2C, Figure EV4.*

2. The authors provide Ki67 stainings to demonstrate the hyperplasic response to H. Pylori infection in the various genetic mouse models utilized. The number of Ki67+ cells will however need to be quantified and normalized to total cell numbers across experimental setups in infected controls, infected experimental and non-infected controls before the effect of H. Pylori infection on tissue hyperplasia can be appreciated.

> *Ki67 has now been quantified and normalized to total cell numbers. The results show that infection upregulates Ki67 in WT and Lgr5 KO mice, but not in Lgr4 KO mice (Figure 1, Figure 2).*

3. Along similar lines, the expression level of p65 in figure 3G will need to be quantified and normalized to DAPI or similar in non-infected controls, infected controls and infected experimental animals to appreciate the effect of genetic perturbations and infection on p65 expression levels. Supporting data in the form of western blotting or similar would further strengthen findings from IHC quantifications.

> *We have now performed several new experiments to further explore the Rspo-NF-kB connection:*
> *- via immunofluorescence and staining for nuclear p65: p65 is present predominantly in the Ki67+ isthmus and (to a lesser degree) in gland base secretory cells with downregulation upon Lgr4 KO in vivo (Figure 3E, F)*
> *- via immunofluorescence: Localization of Cxcl1 and the active form of p65 (K310) predominantly in isthmus cells in vivo (Figure 3G, I).*
> *-via electrophoretic mobility shift assay (EMSA): NF-kB binding capacity is dependent on Rspo in organoids (Figure 7A, B).*
> *- via Supershift analysis in organoids: we demonstrated that the canonical NF-kB pathway is activated by ADP heptose and repressed upon Rspo removal, and that specifically the p65 subunit is responsible for this activation (Figure 7C).*
> *- k-EGFP reporter mouse and organoids: Localization of nuclear p65 in proliferative and in a subset of gland base secretory cells in organoids (Figure EV5A) as well as localization of active NF-kB in organoids using a NF-kB reporter mouse line (EGFP) (Figure EV5B, C).*

4. In figure 4 the authors use qPCR to investigate the role of ADP-heptose and supposed NF-KB signaling activation in epithelial organoids in various medium compositions. The interpretation of the effect of ADP-heptose treatment in the various medias is however difficult as all displayed data is normalized to full medium control. Given that the expression level of a number of targets in various media is displayed in figure 5A, it could help data interpretation if the data in figure 4C and D was normalized to the non-ADP-heptose/TNF-alpha treated controls within individual medias.

> *This has been changed (Figure 6E, F).*

5. In addition to the above, it would strengthen the evidence of ADP-heptose signaling functioning via NF-KB to display IF images of p65 with supposed nuclear localization in FM + ADP-heptose versus FM controls.

> *We have now performed labeling for p65 (Figure EV5A). Importantly, we have now also performed EMSA that shows strong activation of NF-kB binding activity in FM + ADP heptose organoids (Figure 7A, B).*

6. The authors utilize a genetic mouse model with repressed NF-KB function to investigate the role of the signaling pathway in differentiation of secretory cells with protective roles during H. Pylori infection. However, it is not clear to this reviewer how this conditional superrepressor model was implemented exactly. Which Cre-driver was used to activate expression of the superrepressor?

> *We have now explained this mouse model in more detail: A transdominant negative mutant of IkBa (IkBaΔN) was inserted in-frame into the beta catenin locus, allowing ubiquitous expression IkBaΔN. Thus, the phenotype observed here is a result of ubiquitous suppression of NF-kB activity. Importantly, the beta catenin protein is not impaired (Schmidt-Ullrich 2001).*
>
> *Ll. 257-261 "Therefore, we obtained mice with ubiquitously suppressed NF-κB activity, by using a transdominant negative mutant of IkBa (IkBaΔN) that was inserted in-frame into the beta-catenin locus (cI kappa B alpha Delta N, hereafter referred to as ΔN). Importantly, the beta-catenin protein expression and activity in these mice is not impaired (Schmidt-Ullrich et al, 2001)."*

7. The authors hypothesize that a conserved response in gland/crypt bases throughout the gastrointestinal tract might have evolved to sense microbial colonization in this niche. It could certainly be interesting to follow up this notion by investigating whether systemic exogenous Rspo delivery could elicit such a conserved response in the form of p65 upregulation across glands in the GI tract.

This is an interesting idea, which we followed up by infecting mice that conditionally overexpress Rspo3 in Myh11+ cells (Myh11CreERT2/Rosa26Sor6(CAG-Rspo3) – Rspo3 knock-in) with H. pylori for 6 weeks. These mice did indeed show upregulation of p65 (Figure 4E-I). It would be indeed interesting to explore the responses to intestinal pathogens in these mice, which we so far did not explore, and which was not possible within the scope of this paper. However, we do discuss this idea: Ll. 313-316 "It is likely that this unique ability of gland and crypt base cells is not restricted to H. pylori infection in the stomach. Glands and crypts show a similar organization throughout the gastrointestinal tract, with stem cells located in the base. It will be interesting to investigate mechanisms of Rspo-NF-κB interactions in response to intestinal pathogens."

Given that these above major and minor concerns can be addressed to justify the conclusions drawn from the presented data, the findings and conclusions could be of interest for the readership of The EMBO Journal.

Dear Dr. Sigal,

Thank you for submitting your revised manuscript (EMBOJ-2021-109996R) to The EMBO Journal. Your amended study was sent back to the three referees for their re-evaluation, and we have received comments from all of them, which I enclose below. As you will see, the reviewers stated that their issues have been comprehensively resolved and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining points of referee #3 carefully, and address these by adding complementary data or revising text and data presentation where appropriate.

In addition, we need you to take care of a number of minor issues related to formatting and data presentation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you may have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure that summarizes the article. I would appreciate if you could provide this figure.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>>Please adjust the title of the 'Conflict of Interests' section to 'Disclosure and Competing Interests Statement'.

>> Introduce an http link for the GEO specifier in the data accessibility section for the microarray dataset generated in this study; remove the referee token and release privacy.

>> Check and in case update publication status of the bioRxiv entry (Sergushichev et al, 2016) in the reference list.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (24th Jul 2022). Please discuss the revision progress ahead of this time with

the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

No additional concerns. This is a revised manuscript, and all of my concerns from the initial review have been addressed. The manuscript is much improved and will be an impactful, highly cited publication.

Referee #2:

The authors have done extensive revisions to address the many comments of the reviewers. I have no further comments. This is a very nice work.

Referee #3:

Revised edition of Wizenty et al., 2022, EMBO Journal:
Following the revision phase, the authors have managed to improve the manuscript by implementing several suggestions and additional experiments as requested by the reviewers. Several unresolved questions however remain which will need to be addressed before the manuscript meets the standards of the EMBO journal. Below I will comment on the authors' replies to the concerns raised in my initial report and state whether they have been acceptably addressed:

Major concerns:
1. The authors have adjusted the interpretations of results throughout the manuscript to explicitly state that the loss of Lgr5 expression in Lgr4 KO animals hampers the specific interpretation of the unique role of Lgr4 in the system. Due to the absence of a phenotype in the Lgr5 KO animals and the adjusted text where the unique role of Lgr4 in mediating the response to H. Pylori infection has been toned down, this serves as a sufficient cautious interpretation of the data presented and hence this concern seems acceptably addressed.

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3. The authors have addressed the infiltration of a series of inflammatory cell types and found a functional consequence of the diminished epithelial response to infection in the Lgr4 KO model, namely the reduced infiltration of neutrophils likely due to the initial finding of reduced expression of neutrophil attractive chemokines. However, as these examinations are performed 2 months after infiltration, and neutrophils are usually associated with an acute inflammatory response (1-2 days after infection) while macrophages and T-cells are associated with chronic inflammation, as would be expected in the case of H. Pylori infection, the authors need to elaborate on the role of epithelial Lgr4 expression in this system. Why does the increased H. Pylori colonization in the Lgr4KO animals compared to controls not lead to excessive infiltration of leukocytes (macrophages and T-cells)? Is the role of the uncovered Rspo-Lgr4-NF-KB axis solely relying on attracting neutrophils to prevent the early phase of gland colonization, and how is this acute phase inflammation envisioned to be occurring after 2 months of infection where the infiltration phenotype of neutrophils is uncovered?

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This has been edited. Privacy was released.

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We would like to thank the Referee #1 and Referee #2 for their valuable comments.

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colonization. Can the authors provide similar images in the Lgr5 model and potentially also quantify the extent of gland colonization across models?

Gland colonization was observed in both Lgr4 KO and Lgr5 KO. We have added an image for Lgr5 KO now (Figure EV2A).

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Indeed, these points should be considered, and we now discuss this in more detail. H. pylori infection leads to a "chronic active gastritis" which is characterized by infiltration of inflammatory cells, with neutrophils being an important and relevant cell population, responsible for the degree of inflammation (e.g. PMID: 11091274). Our data reveal that in this chronic active inflammation neutrophils are regulated by Lgr4. The concept of "chronic active gastritis" and the role of neutrophils based on previous data has now been implemented in the manuscript.

Several studies have reported that the degree of inflammation in mice upon infection with H. plyori is negatively correlated with colonization (Arnold, Gastroenterology, 2011; Wunder, Nature Medicine, 2006), which is in line with our observation of no excessive infiltration of leukocytes in Lgr4 KO animals.

Ll. 322-329 "Infection causes a chronic active gastritis, characterized by mucosal infiltration of immune cells such as neutrophils (Eck et al, 2000), that can progress to atrophic or hyperplastic gastritis, metaplasia and, in about 1% of patients, gastric cancer (Correa, 1995). Neutrophils are associated with mucosal damage in H. pylori gastritis (Ernst et al, 1997; Shimoyama & Crabtree, 1998). It is well established that the inflammatory response to infection is linked to epithelial pathology and mouse experiments have demonstrated that the degree of immune responses against H. pylori correlates with the degree of epithelial premalignant pathology, while the degree of inflammation correlates negatively with H. pylori colonization (Arnold et al, 2011; Wunder et al, 2006)."

Ll. 334.337 "Here, we demonstrate and mechanistically resolve the unique ability of the epithelial gland base module to initiate a pro-inflammatory NF-κB response that promotes an accumulation of gland base cells and simultaneously induces an upregulation of epithelial chemokines leading to mucosal neutrophil infiltration that can initiate and maintain a chronic active gastritis."

Minor concerns:
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This has been changed (Fig 3G, Fig 4I).

b. A brief description of how the EMSA assay was carried out in the current manuscript would be beneficial, as it is hard to appreciate the value of the data in its current state despite the references for the protocols in the methods section

An EMSA description has been added.

Ll. 542-553 "The gel electrophoresis mobility shift assay (EMSA) is used to detect protein complexes with nucleic acids. Here, EMSA was used to determine the affinity of NF-κB to the specific sequence on DNA (the κB sequence). EMSA provides a readout of the activation state of the NF-κB pathway. The 32p radiolabeled DNA probe containing the κB sequence was incubated with the protein lysate and then separated on a gel. EMSA was performed with whole-cell lysates (WCL). Whole-cell lysates were prepared using Baeuerle buffer (20 mM HEPES pH 7.9; 350 mM NaCl; 20% glycerol; 1 mM MgCl2; 0.5 mM EDTA; 0.1 mM EGTA; 1% NP-40), including complete protease inhibitor cocktail (Roche), 10 mM NaF, 8 mM β-glycerophosphate, 0.2 mM Na3VO4, 1 mM DTT. H2KκB probe from MHC promoter was used and incubated with 5 ug of whole cell lysate for 30 min. The protein and DNA was then separated on a non-denaturing TBE-polyacrylamide gel over 90 minutes. The gel was vacuum dried, and signal was visualized on an X ray film."

4. The authors have addressed this issue
5. Could the authors add arrows/arrowheads to the cells in which they believe to observe nuclear p65 staining? An image with p65 and DAPI could also help this interpretation.

We have added arrows to cells with nuclear p65 staining and provided an image with p65 and DAPI (Fig EV5A).

6. The authors have addressed this issue
7. The authors have impressively performed an experiment in a, to this manuscript, new genetic mouse model. Here the authors should display images of p65 in uninfected controls and uninfected KI animals for reference. Did the KI of Rspo3 lead to enhanced protection against H. Pylori compared to controls, e.g. in the number of CFU/g tissue between models? If the Myh11-Cre model is also active in other regions of the gastrointestinal tract, it would add value to investigate p65 expression in glands from such tissues as well (vs controls, no infection model), to support the hypothesis of a conserved role of the Rspo-Lgr-NF-KB axis in protection against pathogens by setting a baseline of NF-KB expression.

We have added images of p65 in uninfected controls and uninfected KI animals (Fig. 4E-H). Indeed, the Rspo3 KI counterbalances H. pylori colonization and gland infiltration, which we described earlier and cite now (Sigal, Nature Cell Biology, 2019). We have added data on p65 expression in the colon of Rspo KI animals (vs controls) to support the hypothesis of a conserved baseline NF-κB expression (Fig EV3B).

Ll. 72-76 "We have previously shown that Rspo3 counterbalances colonization and gland infiltration of H. pylori and mechanistically drives expansion of rapidly cycling Axin2+/Lgr4+/Lgr5- cells in the isthmus, as well as slowly cycling Axin2+/Lgr4+Lgr5+ basal cells that differentiate into secretory cells leading to hyperplasia in the antrum (Sigal et al., 2017; Sigal et al, 2019; Sigal et al., 2015)."

Suggested text edits:

L41: word -> worlds.
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All points have been edited.

2nd Revision - Editorial Decision **17th May 2022**

Dear Dr Sigal,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figure included in this file.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
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https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Please consider us again in the future for your most exciting work.

Best regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
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EMBO Press Author Checklist

Corresponding Author Name: Michael Sigal
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-109996

USEFUL LINKS FOR COMPLETING THIS FORM

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EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Antibodies are listed in Methods.

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Primer sequences are provided in Methods.

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Primary cultures obtained from mouse tissue are explained in Methods.
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Laboratory animals are listed in Methods.
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Housing conditions are available in Methods.

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Helicobacter pylori species are explained in Methods.

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	The histopathology service is mentioned in acknowledgments.

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	No statistical methods were used to predetermine sample size. The sample size was based on availability of mice with the respective genotype and previous experience
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Animal experiments were done on littermates randomly allocated to different experimental groups (see Methods).
Include a statement about **blinding** even if no blinding was done.	Yes	The investigator was blinded for analysis of CFU counts, image analysis and for quantitative analysis (see Methods).
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	No data were excluded from the experiments.
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	For samples of 3 or more ANOVA with Tukey´s Multiple comparison was used. For studies involving two groups, we used an unpaired two-sided t-test. Assumption of homoscedasticity is assumed for ANOVA and t-test. For transcriptome data, please see Methods.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	See figure legends.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	See figure legends.

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	All aspects of animal care and experimental protocols in this study were approved by the regulatory standards of the Berlin Animal Review Board (LAGeSo Berlin) (Reg. G0084/17) (see Methods).
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Yes	Approval for work Helicobacter pylori specimen was provided in accordance with local laws (BioStoffV and GenTSV).

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	We confirm compliance with ARRIVE.
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Microarray data have been deposited to the GEO database. The accession number is provided in the Data Availability Section.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	The respective data citation is in the reference list.