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Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells ex vivo

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Transaction Report:

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

1st Editorial Decision 30 November 2010

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, while all the referees agree that the study is potentially interesting, they also all raise concerns and make suggestions of how it should be improved. The two main concerns, raised by referees 1 and 2, are that it needs to be ruled out that the loss of stem cells from ex vivo cultures from Lgr4 KO cells is due to defective Paneth cells (instead of reduced Wnt signaling), and that additional evidence is required to support the hypothesis that Lgr4 regulates Wnt signaling. To address these concerns it should be examined whether Paneth cells are present in ex vivo cultures from Lgr4 KO cells and whether Wnt target gene expression is reduced in the intestine of Lgr4 KO mice. Both referees 2 and 3 further indicate that activation of the Wnt pathway, either in vivo or in the cultures, should rescue the Lgr4 KO phenotype. Referee 3 adds that it should be determined whether Lgr4 is expressed in Paneth cells, and along these lines, both referees 2 and 3 remark that Lgr4 expression needs to be documented at higher resolution. The referees also point out that the description of the experimental set up lacks important details.

Given these evaluations and the constructive referee comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.
I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

RE: Manuscript EMBOR-2010-34557V1

In the Manuscript entitled "Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells" Mustata et al. use a LacZ genetrap knocked in the Lgr4 gene mouse model to analyze the phenotype in the intestine of mice with 10% residual expression of Lgr4 "in vivo" and in an ex-vivo crypt growth system. They show that Lgr4 is expressed in inter villus pockets in newborn mice and in intestinal crypts in adults. In vivo, Lgr4 deficiency leads to severe defects in Paneth Cell counts and Paneth Cell differentiation and to a mild decrease in epithelium proliferation. Shortage of Lgr4 has a dramatic effect on crypt growth/survival in ex-vivo cultures of crypts: 2% growth of Lgr4 KOs crypts vs 58-47% in WT or heterozygous crypts respectively. Microarray experiments on ex vivo crypts from WT and Lgr4 KOs show downregulation of stem cell markers/wnt targets.

Given the similarities between the "in vivo" phenotype of Lgr4 KO with that of mouse hypomorphic for beta-catenin, together with the ex vivo experiments and microarray results, the authors propose that Lgr4 functions to positively regulate Wnt signaling in intestinal crypts.

The data obtained both in vivo and in the ex vivo system are robust, evident, and well described although some experiments need more detailed description (see below). While the phenotypes observed are indeed compatible with a decrease in Wnt signalling as proposed by the authors, this reviewer's only concern is how to reconcile the dramatic defects seen in the "ex vivo" culture system with the very mild phenotype observed "in vivo" regarding crypt maintenance (see below). In addition, recovery of plating efficiency by Wnt stimulators is rather poor in Lgr4 KO crypts (negative for R-spondin and 6% with LiCl) suggesting that stimulating Wnt signaling in Lgr4KO ex vivo crypts is not sufficient to compensate for Lgr4 deficiency.

Hans Clevers' recently reported that for efficient plating and organoid growth, intestinal stem cells required to be associated with Paneth Cells (Snipperts et al, Cell 2010). Authors should rule out that the ex-vivo cultures from Lgr4 KOs reflect the defects observed in Paneth cell's function/numbers. They argue that defective Paneth cell differentiation may not be the cause of cell death as the phenotype of newborn mice -where Paneth cells are not present- is the same as for P15 mice. However, mRNA for cryptidins 1 and 6 can be readily detected at stage E14.5 of development, suggesting that even if not present as mature Paneth cells, functional precursors may exist (van Es et al., Nat Cell Biol 2005).

Thus, it would be important to strengthen the point whether loss of stem cell markers/wnt targets or stem cells is due to diminished Wnt signaling in stem cells or whether defective/deficient Paneth cells in Lgr4 KO ex-vivo crypts are unable to support Stem Cell survival through some alternative mechanism.

Specific Points:

- Please clarify whether Paneth cells are present or not in ex- vivo growth experiments and microarrays. Please state clearly in the manuscript whether microarray experiments (supplemental Table S1) are carried out using crypts from P0 or P15 mice. At Day 0, classical Wnt targets Axin2, myc, Lgr5, sox9 for example are expressed at fairly similar levels, suggesting similar degree of Wnt signaling in both situations. Yet, Paneth cell markers are dramatically downregulated (10-30 fold),
suggesting Paneth cells present in WT crypts but absent from KOs could contribute to ex vivo crypt growth/survival.

- If microarray analysis were done from crypts at P15, then please demonstrate by qPCR, microarrays or any alternative means whether there are Paneth cells or not in P0 ex-vivo crypts.

- Would it be possible to culture single crypt stem cells from Lgr4KOs with Paneth cells from WT mouse or Paneth cells from Lgr4KO mice? This would illustrate cross-talk between Paneth cells and intestinal stem cells. I realize this may be tricky.

- What is the ex-vivo growth/survival of beta-catenin hypomorphic crypts?

- Could authors compare Wnt target gene expression by qPCR in p15 intestines of Lgr4KOs and WT as in Garcia et al, 2009? Axin2 and Acs12 would be particularly informative regarding Wnt signaling levels. This data would support author's claim of diminished Wnt signaling in crypts.

- Usually inhibition of Wnt signaling is accompanied by differentiation of intestinal epithelial cells. It is somewhat surprising that differentiation of enterocytes, mucosecreting and enteroendocrine cells in vivo is not affected in Lgr4 KOs. This is difficult to appreciate from Supplemental Figure S1. Immunofluorescence panels in Fig S1A are small, dark and difficult to evaluate. Alcian blue stainings should be evaluated in longitudinal sections of crypts.

- The differences between growth/survival of Lgr4 and Lgr5 deficient crypts are rather interesting. How do authors reconcile these antagonistic functions? Lgr5KO show increases in Wnt signaling, whereas Lgr4KO, according to authors, show downmodulation of Wnt signaling. Are Lgr4 and Lgr5 expression patterns in intestinal crypts or along the intestinal tube mutually exclusive? Do they fine-tune Wnt signaling in cells depending on what Lgr molecule is expressed at a certain time-space point? What is the model author's propose? Please discuss.

Minor points

- WT panels in Figure 1 should be equal size as LacZ. For reviewing purposes it would be advisable to present larger panels. In general, Figure legends/methods/ or text should specify in more detail the experiments carried out. For example it is unclear whether Figure 1D and E correspond to adult tissue.

- Since ISH for Lgr4 works fairly well, it would be advisable to include ISH results to ensure that Lgr4 mRNA expression pattern follows that of LacZ. Particularly in light of the results by Mazebourg et al, 2004, which show immunostaining in neonatal villus

- If Fig1D states Lgr4 is expressed in subepithelial fibs? What is the criteria to define them as such? It is difficult to ascribe a phenotype to the scattered blue nuclei observed in the bottom panel of Fig 1D.

- Please state clearly in the text and figure legends whether ex vivo experiments are carried out from whole crypts or individual cells and from mice at what stage. For example in Figure 3D it is unclear from text and figure legend whether panels correspond to P0 or P11 mice or which ones to what.

- Please include Paneth cell markers in Fig 3E

Referee #2 (Revision Comments):

This manuscript by Mustata et al. describes the expression pattern and addresses the function of the Lgr4 receptor in the small intestine using mice with a hypomorphic Lgr4 allele and crypt cultures derived from them. Lgr receptors have recently received a lot of attention as stem cell markers in various morphological structures, but data on their functions are still scarce. The issue is thus important and timely.

Major comments:

1-The pictures selected to illustrate the Lgr4 expression pattern using the LacZ reporter suffer from
poor resolution (Figs 1B,C,E).

2-The conclusion that Lgr4 regulates Wnt signalling is based on limited, mostly indirect evidence and appears overstated in the manuscript. Additional data is needed to support the statement.

Specific comments

Figures 1A to 1C: rare LacZ expressing cells in the mesenchyme and smooth muscle at P0 (1A), but much more staining in the adult smooth muscle. Is this specific?

Fig 1D (top): the authors should provide a better image of a well-oriented crypt section at higher magnification.

10% Lgr4 expression in the Lgr4KO: the authors should indicate what kind of Lgr4 transcript is generated in the Lgr4KO mice. Is this supposed to be functional?

Crypts are shorter in Lgr4KO mice, and these animals also have fewer Paneth cells. While the authors rule out that Lgr4 deficiency could cause a delay in the onset of ileum differentiation because all other lineages are unaffected, it would be important to check that there is no delay in the morphogenesis of the crypts (for example include two additional time points, P7, P10), since morphologically recognizable Paneth cells appear simultaneously with crypt formation.

In addition, the authors should stain Lgr4KO/control mouse sections and organoids for beta-catenin to support their conclusion that Lgr4 positively regulates Wnt signalling.

That the Lgr4KO/Lgr5KO phenotype is milder than the Lgr4KO phenotype is indeed surprising. As an explanation the authors propose an antagonistic effect of both receptors but alternative possibilities exist, including differences in the genetic background of the Lgr4KO and Lgr4KO/Lgr5KO strains, which is not documented in the manuscript. This information should be available.

The authors could strengthen their conclusion that the collapse of Lgr4KO ex vivo cultures is due to loss of Wnt signalling by adding a Wnt ligand in the culture medium.

Referee #3 (Revision Comments):

In my opinion, this report could be considered for publication if the authors can strengthen some of their results, as suggested in the report.

Referee #3 (Remarks to the Author):

Mustata and colleagues report some interesting findings on the effect of Lgr4 deletion in the mouse small intestine. They find that Lgr4 is essential for Paneth cell differentiation in vivo and for stem cell maintenance ex vivo. The authors suggest that Lgr4 acts as a positive regulator of Wnt signaling in the intestine.

These findings could potentially have important therapeutic implications, though a possible role for Lgr4 in malignancies still remain to be assessed. In addition, the elegant genetic analysis on compound mutant mice shows that Lgr4 is dominant over Lgr5 and that indeed, unlike Lgr5 whose function is dispensable, Lgr4 appears to have an essential role in stem cell survival in organ cultures ex vivo.

General Remarks:

1) The authors clearly show that Lgr4 is required for the proper specification of Paneth cells. In Fig.1D, though, it appears that Lgr4 is NOT expressed in Paneth cells, possibly suggesting a
paracrine effect of this receptor. The authors are invited to unambiguously establish if Lgr4 is expressed in Paneth cells and if this is not the case, to formulate a hypothesis on the non-cell autonomous function of Lgr4.

2) It is unclear if the transcriptional changes in gene expression found in Lgr4 KO vs. WT mice are a consequence of a direct regulation of Lgr4, specifically on Wnt target genes, or if they are secondary to the KO phenotypes, i.e. reduction of Paneth and stem cell markers. A more ambitious experiment that would help clarify the mechanism underlying the effects of Lgr4 in vivo would be to cross Lgr4 KO mice to mutant mice expressing active beta-catenin (Harada et al. EMBO J, 1999) and test if this rescues the loss of Paneth and stem cells. Being aware of the difficulties of such an experiment, though, I would not consider it indispensable for publication.

3) Since Lgr5 is a Wnt target gene itself, it could be interesting to test if Wnt modulation can also affect the levels of Lgr4.

Specific Remarks:

a) The authors are invited to provide a bigger magnification for Fig.1A, since Lgr4 expression in the inter villus pockets of newborn mice is not obvious in the present picture.

b) In Fig.1E, the villi should be shown as well, in order to appreciate the crypt-restricted expression of Lgr4.

c) The number of BrdU positive cells in Fig.1C should be normalized to the total number of crypt cells; since Fig.1A and B clearly show a reduced number of cells per crypt, this would lead to a reduced number of BrdU+ cells per crypt even if Lgr4 had no effect on crypt proliferation.

d) The size of the scale bars in Fig.3 and 4 is missing and should be included in the Figure Legends.

e) The authors are invited to provide a qRT-PCR or better a Northern blot showing the 10% residual expression of Lgr4 in the KO mice.

f) It is unclear if Lgr4 expression as well as its phenotypes are exclusively observed in the ileum or are found in other regions of the small intestine and the colon. X-gal staining on the different intestinal tracts of the Genetrap LacZ knock-in allele is needed, along with an evaluation of the reduction of Paneth cells and proliferating cells in the other regions of the small intestine of Lgr4-/- mice.

g) The authors find by microarray analysis that, with the exception of Paneth cell specific genes, markers of terminal lineage differentiation are upregulated in Lgr4 KO organoids. It is not shown, though, if this reflects an increase in the number of differentiated cells in the "minigut" cultures coming from KO mice. Staining for those specific markers in the organoids from KO and WT mice would clarify this point.

1st Revision - authors' response 10 November 2010

We thank the editor and the referees for their overall positive evaluation of our manuscript and for their suggestions for improvement.

We have carefully considered all points raised, performed additional experiments and provide hereunder a detailed account of how we have handled them.

Answers to the main concerns as summarized by the editor.

The two main concerns, raised by referees 1 and 2, are that it needs to be ruled out that the loss of stem cells from ex vivo cultures from Lgr4 KO cells is due to defective Paneth cells (instead of reduced Wnt signaling), and that additional evidence is required to support the hypothesis that Lgr4 regulates Wnt signaling.... Both referees 2 and 3 further indicate that activation of the Wnt pathway, either in vivo or in the cultures, should rescue the Lgr4 KO phenotype.
We totally agree that this point has become crucial, even more so, after publication by the Clevers’ group (coming out after our ms had been reviewed) that Wnt ligands (Wnt3 and Wnt11) produced by Paneth cells are required to sustain ex vivo development of organoids from single CBC cells (Sato et al Nature vol 469, 415-418, 2011). In this paper, authors show that addition of Wnt3a to the culture of single CBCs can compensate for the absence of Paneth cells and that Paneth cells contribute to maintain Lgr5 positive stem cells in vivo.

We have addressed this point in two different ways:

- By assaying by qRT-PCR a series of additional Paneth-specific transcripts including Wnt3 and Wnt11 (qRT-PCR) in crypts at the time of starting the ex vivo cultures (day 0). Whereas markers of terminal Paneth cell differentiation (cryptdin4 and lysozyme) are strongly downregulated in Lgr4KO crypts (~10fold), Wnt3, Wnt11, Mmp7 and Egf transcripts are at the same levels as in WT crypts. We conclude that lack of Lgr4, in vivo, causes a blockade in terminal differentiaation of Paneth cells, but is compatible with production of Wnt agonists at normal levels. These results strongly suggest that the loss of CBC cells and subsequent death of organoids, ex vivo, is not due to a lack of Wnt ligands.

- By attempting to rescue Lgr4KO crypts ex vivo by addition of Wnt3a (same concentration as in Sato et al. 2011) or with a series of low molecular weight inhibitors of gsk3b or Wnt agonists. While we still observe partial rescue with LiCl, no rescue was observed with Wnt3a or any of these agents, although they were clearly able to stimulate canonical Wnt signaling in the C2C12 cell differentiation assay or in HEK293 cells expressing the TOP-flash reporter.

We have included these key novel data in figures 3F and Supplementary Table 3. These observations are totally compatible with the original ones but provide further understanding regarding the effect of Lgr4 on Wnt signaling. Since lack of Lgr4 ex vivo cannot be rescued by Wnt or Wnt agonists, the observed downregulation of Wnt targets and death of stem cells ex vivo means that Lgr4 does not directly activate Wnt signaling but, rather, plays a permissive role on the Wnt pathway in CBCs, or on stem cell survival. Moreover, the LiCl rescuing effect cannot be related to the mere inhibition of gsk3b since other GSK3b inhibitors do not efficiently rescue KO crypts. Therefore, LiCl does likely mimic, at least partially, the permissive effect of Lgr4. We have modified the discussion of our results to account for the new data. Although we would love to unravel the mechanism of LiCl action, we consider that this is beyond the scope of the present revision.

….. it should be examined whether Paneth cells are present in ex vivo cultures from Lgr4 KO cells....

Paneth cells are definitely present morphologically in P15 Lgr4KO crypts (Lendrum staining) in vivo, but at a very low level (15% of WT) and with immature characteristics (Fig.2D and Supplementary fig.2C). Accordingly, Paneth cell markers are present in Lgr4KO ex vivo cultures at the time of seeding, but the level of their transcripts depends on the nature of the marker. Whereas lysozyme and cryptdin4 transcripts are about 10 fold lower than in WT crypts, MMP7, Wnt3, Wnt11 and Egf are at the same level as in WT. As mentioned above, this is compatible with an effect of Lgr4 deficiency on the terminal differentiation of Paneth cells, in vivo. These data have been added to fig.3F.

…. and whether Wnt target gene expression is reduced in the intestine of Lgr4 KO mice.

In our original figure 3E, we showed that CBC markers and Wnt target genes (Lgr5, Ascl2, Axin2, Sox9…) are not downregulated in Lgr4KO crypts at the time of seeding (day 0), while their expression decreases dramatically thereafter. In the new Fig. 3F, we show that Wnt ligand transcripts are not downregulated, neither, at day 0. This indicates that Wnt signals are maintained in Lgr4KO crypts in vivo. Interestingly, Wnt3 and Wnt11 transcripts decrease much more slowly than CBC markers (Lgr5, Ascl2…) during the first day of culture (new Fig.3E,F), suggesting that the death of CBCs is not a consequence of the lack of Wnt ligands. Together these observations suggest that Wnt-permissive signals, or CBC survival factors, either systemic, or originating from the surrounding tissues (mesenchyme, muscle…) compensate for Lgr4 deficiency, in vivo, and that these signals are acutely missing upon culturing (i.e. they are not made by the crypts themselves and/or are not present in the medium). Given the documented role of Wnt signaling on survival and
proliferation of CBCs, and on their differentiation into Paneth cells, we favor the hypothesis of a permissive effect of Lgr4 on the Wnt pathway. The text has been modified to include the above data and discussion.

Referee 3 adds that it should be determined whether Lgr4 is expressed in Paneth cells, and along these lines, both referees 2 and 3 remark that Lgr4 expression needs to be documented at higher resolution.

Fig. 1 showing Lgr4 expression in the small intestine has been completely modified with new pictures on X-gal staining and in situ hybridization. Moreover, we have examined in more detail whether Lgr4 is expressed or not in Paneth cells. Immunofluorescence experiments with anti-bgal and anti-P-lyz antibodies strongly suggest that Lgr4 is actually expressed in a minority of Paneth cells (see new Figure 1C). This is compatible with the hypothesis that lack of Lgr4 would affect Paneth cell precursors cell-autonomously and be responsible for the blockade of their terminal differentiation. Besides, we have documented again expression of Lgr4 in the smooth muscle and mesenchymal cells. Double immunofluorescence with anti-b-gal and Vimentin antibodies show that, at least part of the mesenchymal Lgr4-expressing cells, correspond to subepithelial myofibroblasts (new Supplementary Fig. S1B).

The referees also point out that the description of the experimental set up lacks important details.

We have answered their specific queries (see below).

Answers to referee #1.
Please read also the “Answers to the main concerns as summarized by the editor”, above.

While the phenotypes observed are indeed compatible with a decrease in Wnt signaling as proposed by the authors, this reviewer's only concern is how to reconcile the dramatic defects seen in the "ex vivo" culture system with the very mild phenotype observed "in vivo" regarding crypt maintenance....

.... Thus, it would be important to strengthen the point whether loss of stem cell markers/wnt targets or stem cells is due to diminished Wnt signaling in stem cells or whether defective/deficient Paneth cells in Lgr4 KO ex-vivo crypts are unable to support Stem Cell survival through some alternative mechanism

This key question has been addressed in detail in the “Answers to the main concerns”, see above.

Specific Points

- Please clarify whether Paneth cells are present or not in ex-vivo growth experiments and microarrays.

Paneth cells are definitely present morphologically in P15 Lgr4KO crypts (Lendrum staining) in vivo, but at a very low level (15% of WT) and with immature characteristics (Fig.2D and Supplementary fig.2C). Accordingly, Paneth cell markers are present in Lgr4KO ex vivo cultures at the time of seeding, but the level of their transcripts depends on the nature of the marker. Whereas lysozyme and cryptdin4 transcripts are about 10 fold lower than in WT crypts, MMP7, Wnt3, Wnt11 and Egf are at the same level as in WT. As mentioned above, this is compatible with an effect of Lgr4 deficiency on the terminal differentiation of Paneth cells, in vivo. These data have been added to fig.3F.

Please state clearly in the manuscript whether microarray experiments (supplemental Table S1) are carried out using crypts from P0 or P15 mice.

Sorry for the omission. The results are from P15 mice. This information has been added to the legend of the supplementary table S1 and in the text (materials and methods).

At Day 0, classical Wnt targets Axin2, myc, Lgr5, sox9 for example are expressed at fairly similar levels, suggesting similar degree of Wnt signaling in both situations. Yet, Paneth cell markers are dramatically downregulated (10-30 fold), suggesting Paneth cells present in WT crypts but absent
from KOs could contribute to ex vivo crypt growth/survival.
This point has been addressed by new experiments as described in the “answers to the main concerns”, above.

It is an important point which was not satisfactorily discussed in the original manuscript. Indeed, if defective Wnt signaling would be responsible for the Lgr4KO phenotype, how could it be that, in vivo, it would cause loss of Paneth cell differentiation, while simultaneously be compatible with maintenance of crypts/CBCs?

Our current interpretation, coming from the new results (measure of Wnt3, Wnt11 transcripts at day 0 of culture, absence of rescue by exogenous Wnt3a addition; see above), is that Lgr4 signaling would be permissive for the Wnt pathway rather than activate it and would be required cell-autonomously for the terminal differentiation of Paneth cells (see above). This implies that a substitute of Lgr4 must be active in Lgr4KOs, in vivo, to account for survival of crypts/CBCs, but that this substitute would not allow for terminal differentiation of Paneth cells. We have attempted summarizing our current interpretation of the data in a model presented as an annex to this rebuttal (see annex 1). Unfortunately, the space limitation does not allow inclusion of this model as an additional figure, but we have clearly discussed its content in our revised manuscript.

- If microarray analysis were done from crypts at P15, then please demonstrate by qPCR, microarrays or any alternative means whether there are Paneth cells or not in P0 ex-vivo crypts. There is obviously no mature Paneth cell in the intestine of newborn mice. Nevertheless, some Paneth cell transcripts are found by qRT-PCR at P0 in both WT and Lgr4KO and can be localized to the inter villus regions by ISH using a Crypt4 riboprobe (new Supplementary Fig.S3B). Therefore, the death of Lgr4KO P0 progenitors upon ex vivo culture cannot be attributed to deficiency in Paneth cell precursors. The origin of the Wnt required to support organoid development from newborn tissue is not clear: Wnt signaling is required for the normal development of crypts and differentiation of Paneth cells from early progenitors, and Paneth cells produce the Wnt required for maintenance of adult stem cells. It is thus likely that Wnt is produced at P0, either by incompletely differentiated Paneth cells, or maybe more likely, in an autocrine manner by progenitors. We have not explored this further at this time.

- Would it be possible to culture single crypt stem cells from Lgr4KOs with Paneth cells from WT mouse or Paneth cells from Lgr4KO mice? This would illustrate cross-talk between Paneth cells and intestinal stem cells. I realize this may be tricky.

After the paper of Sato et al showing that Wnt3a allows ex vivo crypt development from single CBCs in the absence of Paneth cells (Sato et al Nature vol 469, 415-418, 2011), we think that this kind of (indeed tricky) experiment is not needed anymore, given our observation that Wnt3a or Wnt agonists do not rescue Lgr4KO crypts ex vivo (see above).

- What is the ex-vivo growth/survival of beta-catenin hypomorphic crypts?

Unfortunately, we don’t have access to these mice and could not perform this kind of experiment within the time frame of the resubmission.

- Could authors compare Wnt target gene expression by qPCR in p15 intestines of Lgr4KOs and WT as in Garcia et al, 2009. Axin2 and Acsl2 would be particularly informative regarding Wnt signaling levels. This data would support author's claim of diminished Wnt signaling in crypts. Since Acsl2, Lgr5 and Axin2 are essentially expressed in crypts (in CBCs), we consider that the qRT-PCR performed at day 0 on the material used for seeding the cultures reflects the situation in the intestine (new Fig.3E). As discussed above, there is no sign of decreased Wnt signaling from these markers, at the time of seeding (day 0), while there is dramatic down-regulation already after 12h of culture. This fits completely with our current interpretation described above.

- Usually inhibition of Wnt signaling is accompanied by differentiation of intestinal epithelial cells. It is somewhat surprising that differentiation of enterocytes, mucosecreting and enteroendocrine cells in vivo is not affected in Lgr4 KOx. This is difficult to appreciate from Supplemental Figure S1. Immunofluorescence panels in Fig S1A are small, dark and difficult to evaluate. Alcian blue stainings should be evaluated in longitudinal sections of crypts.
We agree that this was not easy to reconcile with inhibition of Wnt signaling. It fits much better with our current interpretation that Lgr4 has a permissive effect on Wnt signaling, and that its deficiency would be compensated in Lgr5KO, in vivo. Regarding the quality of the Supplementary Fig S1, we have used pseudocolors to improve the contrast of the immunofluorescence pictures and provide new pictures for alcian blue staining, with well-oriented crypts in a new suppl Fig S2B.

- The differences between growth/survival of Lgr4 and Lgr5 deficient crypts are rather interesting. How do authors reconcile these antagonistic functions? Lgr5KO show increases in Wnt signaling, whereas Lgr4KO, according to authors, show downmodulation of Wnt signaling. Are Lgr4 and Lgr5 expression patterns in intestinal crypts or along the intestinal tube mutually exclusive? Do they fine-tune Wnt signaling in cells depending on what Lgr molecule is expressed at a certain time-space point? What is the model author's propose? Please discuss. Although sketchy and with open questions, especially in the absence of knowledge on the Lgr4,5 agonist(s), we propose the following model which accounts for our data (see also the figure in annex 1 to this letter).

Based on qRT-PCR and X-gal staining experiments (see new Fig.1 and Supplementary Fig. S1A), expression of both receptors does not appear timely or spatially exclusive in CBCs. They may be necessary to fine-tune Wnt signaling in epithelial stem cells and TA cells: Lgr5 being required to prevent excessive Wnt signaling in CBCs and Lgr4 being necessary to maintain sufficient Wnt stimulation in both CBCs and TA cells.

- In vivo: lack of Lgr5, acting only in CBCs, would upregulate the Wnt pathway in these cells and lead to premature differentiation of Paneth cells in homozygote KOs (Garcia et al 2009). Stimulation of a regulatory cascade controlled by Lgr4 would be required for Wnt signaling to be effective in CBCs and early Paneth cells. The absence of Lgr4 would be complemented in CBCs by the redundant receptor activated by a putative agonist coming from the mesenchyme/muscle. As LGR4 is normally present in the mesenchyme/muscle, it is conceivable that there would even be an increased production of this agonist in Lgr4KOs. The receptor of the mesenchyme/muscle agonist would be absent in early Paneth cells, thus explaining the block of their terminal differentiation observed in vivo (see annex 1).

- Ex vivo: lack of Lgr5 (data only available at P0 because of neonatal death of homozygotes) is compatible with normal development of organoids. They grow and develop at least as well as WT (Fig 3C). Whether progenitor’s growth relies on Wnt signals originating from Paneth cell precursors (mature Paneth cells are not present at P0 in WT progenitors) or generated in an autocrine way is unknown (see above). In the absence of the putative mesenchyme/muscle-redundant agonist, lack of Lgr4 in Lgr4KOs results in rapid downregulation of the Wnt pathway and death of CBCs, because of the loss of the permissive effect of Lgr4 (Fig3C).

In Lgr4/Lgr5 double KOs, the lack of Lgr4 is dominant, meaning that upregulation of the Wnt pathway in Lgr4KO would be submitted to the permissive effect of Lgr4.

Minor points

- WT panels in Figure 1 should be equal size as LacZ. For reviewing purposes it would be advisable to present larger panels. In general, Figure legends/methods/ or text should specify in more detail the experiments carried out. For example it is unclear whether Figure 1D and E correspond to adult tissue. A novel figure 1 has been prepared according to referee’s comment and the legends made more explicit for all figures.

- Since ISH for Lgr4 works fairly well, it would be advisable to include ISH results to ensure that Lgr4 mRNA expression pattern follows that of LacZ. Particularly in light of the results by Mazerbourg et al, 2004, which show immunostaining in neonatal villus

We have modified fig.1 to better illustrate the specific presence of transcripts in crypts and not in villi (new Fig. 1B). Given that expression of Lgr4 in the neonatal mouse villi were obtained by Mazerbourg et al with an antibody against the human receptor, we think that the burden of the proof...
rests on their shoulders. To allow presentation of the novel data and their discussion, we have removed reference to the Mazerbourg data, in order to save space.

- **Fig1D** states Lgr4 is expressed in subepithelial fibs? What is the criteria to define them as such? It is difficult to ascribe a phenotype to the scattered blue nuclei observed in the bottom panel of Fig 1D.

We have made new immunofluorescence pictures with anti b-gal and Vimentin antibodies. They are displayed in new supplementary Fig. S1B.

- **Please state clearly in the text and figure legends whether ex vivo experiments are carried out from whole crypts or individual cells and from mice at what stage.** For example in Figure 3D it is unclear from text and figure legend whether panels correspond to P0 or P11 mice or which ones to what.

We agree that the text was confusing. Fig 3D corresponds to P11 mice, and 3C to P0. All cultures were started from isolated crypts or, for P0 mice, clumps of multiple cells. This has been clarified in text and Figure legends.

- **Please include Paneth cell markers in Fig 3E**

We have completely modified Fig3E and complemented it by Fig.3F. These combine original qRT-PCR data with results from new experiments. Transcripts corresponding to markers of Paneth cells, Goblet cells, enteroendocrine cells, enterocytes, TAs and CBCs are now illustrated.

**Answers to referee #2.**

Please read also the “Answers to the main concerns as summarized by the editor”, above.

**Major comments**

1. The pictures selected to illustrate the Lgr4 expression pattern using the LacZ reporter suffer from poor resolution (Figs 1B,C,E).

A new version of figure 1 is provided.

2. The conclusion that Lgr4 regulates Wnt signalling is based on limited, mostly indirect evidence and appears overstated in the manuscript. Additional data is needed to support the statement.

The novel data presented in the “Answers to the main concerns as summarized by the editor”, have modified our view of the way Lgr4 regulates Wnt signaling. Please find a detailed presentation of these data and their discussion here above.

**Specific comments**

**Figures 1A to 1C:** rare LacZ expressing cells in the mesenchyme and smooth muscle at P0 (1A), but much more staining in the adult smooth muscle. Is this specific

Although the signals in the mesenchyme and muscle at P0 are weak and not easily displayed in print, they are specific as the sections from WT animals are completely negative. In a new Fig. 1, we have better displayed the negative controls and added results from E15 embryos. In the adult, we observed coherent pictures of Lgr4 expression in muscle using X-gal enzymatic or b-gal immunoreactivity stainings. This is now illustrated in Fig.1A and C.

**Fig 1D (top) :** the authors should provide a better image of a well-oriented crypt section at higher magnification.

A new version of figure 1 is provided.

**10% Lgr4 expression in the Lgr4KO: the authors should indicate what kind of Lgr4 transcript is generated in the Lgr4KO mice. Is this supposed to be functional?**

About 10% of Lgr4 transcripts are indeed present in “Lgr4KOs”, by qRT-PCR. These transcripts are wild-type, corresponding to the splicing-out of the genetrap insert from some of the transcripts together with intron1. It implies ineffective termination of transcription at the polyA addition site of
the construct. Data illustrating partial skipping of the cassette (by RT-PCR) and the residual level of WT transcript (by qRT-PCR) have been added and are presented in supplementary fig S2A.

Crypts are shorter in Lgr4KO mice, and these animals also have fewer Paneth cells. While the authors rule out that Lgr4 deficiency could cause a delay in the onset of ileum differentiation because all other lineages are unaffected, it would be important to check that there is no delay in the morphogenesis of the crypts (for example include two additional time points, P7, P10), since morphologically recognizable Paneth cells appear simultaneously with crypt formation. P7, and P11 data have been added to previously available P15 and P21 data, for Paneth cell counts, BrdU incorporation, crypt depth (see new fig 2B, C and E). They show that crypt formation takes place normally despite defective Paneth cell differentiation.

In addition, the authors should stain Lgr4KO/control mouse sections and organoids for beta-catenin to support their conclusion that Lgr4 positively regulates Wnt signalling. We have observed no difference in beta-catenin labeling between WT and KO crypts (see annex 2 to this letter), or cultured organoids (new supplementary Fig S3C). Nevertheless, we have noticed a strong difference in Ki67 labeling of organoids after 1 day in culture. Whereas the majority of WT nuclei are stained, only rare Ki67 positive nuclei are observed in Lgr4KOs (new supplementary Fig S3C), in agreement with the conclusion that CBCs in Lgr4KO crypts stop dividing and die almost immediately upon culturing. It is likely that Lgr4KO TA cells complete ongoing divisions and start differentiating, as suggested by the increase in all differentiated markers (except those of Paneth cells; see Fig. 3E,F).

That the Lgr4KO/Lgr5KO phenotype is milder than the Lgr4KO phenotype is indeed surprising. As an explanation the authors propose an antagonistic effect of both receptors but alternative possibilities exist, including differences in the genetic background of the Lgr4KO and Lgr4KO/Lgr5KO strains, which is not documented in the manuscript. This information should be available.

The genetic background of Lgr4KOs and Lgr5/Lgr4 double KOs is similar, since the animals used in the experiment documented in Fig 3D are from the same litter. They all result from the mating of double heterozygotes. Actually, the double Lgr4/Lgr5 homozygote knockouts have a similar phenotype ex vivo as the single Lgr4KOs (rapid death of organoids) (Fig 3D), while Lgr5KO organoids survive ex vivo. This justifies our conclusion that lack of Lgr4 is dominant. The opposing effects of Lgr4 and Lgr5 deficiency manifest on Paneth cells [precocious differentiation in Lgr5KO (Garcia et al 2009); defective differentiation in Lgr4KO (present data)] and on neonatal survival of the animals. Lgr5KO mice die during their first day, secondary to ankyloglossia, a phenotype which is not present in Lgr4/Lgr5 double KOs.

The authors could strengthen their conclusion that the collapse of Lgr4KO ex vivo cultures is due to loss of Wnt signalling by adding a Wnt ligand in the culture medium. This has been done, as described in detail under “Answers to the main concerns as summarized by the editor”, above and has been included in the text. Wnt3a addition to the culture medium does not rescue Lgr4KO crypts ex vivo.

Answers to referee #3.

Please read also the “Answers to the main concerns as summarized by the editor”, above.

General Remarks:

1) The authors clearly show that Lgr4 is required for the proper specification of Paneth cells. In Fig 1D, though, it appears that Lgr4 is NOT expressed in Paneth cells, possibly suggesting a paracrine effect of this receptor. The authors are invited to unambiguously establish if Lgr4 is expressed in Paneth cells and if this is not the case, to formulate a hypothesis on the non-cell autonomous function of Lgr4.

We have examined in more detail whether Lgr4 is expressed or not in Paneth cells. Immunofluorescence experiments with anti-bgal and anti-lyz antibodies strongly suggest that Lgr4 is actually expressed in a minority of Paneth cells (see new figure 1C). From these data and the new
results showing a blockade of terminal differentiation of Paneth cells in Lgr4KO crypts (see above), we propose that the effect of Lgr4 deficiency on Paneth cell development is likely cell autonomous, affecting either the potential of CBCs to differentiate into fully mature Paneth cells, or Paneth cell precursors themselves. We have discussed these points in the new version of the manuscript.

2) It is unclear if the transcriptional changes in gene expression found in Lgr4 KO vs. WT mice are a consequence of a direct regulation of Lgr4, specifically on Wnt target genes, or if they are secondary to the KO phenotypes, i.e. reduction of Paneth and stem cell markers.

We think that the new experiments described under “Answers to the main concerns as summarized by the editor” provide clarification. Clearly, it is not the decrease of Wnt normally produced by Paneth cells that is responsible for the ex vivo phenotype. This is shown by the normal levels of Wnt3 and Wnt11 transcripts observed at the initiation of the culture (new Fig.3F) and by the inability of exogenous Wnt3a or Wnt agonists to rescue the Lgr4KO organoids. It suggests that Lgr4 would exert a permissive effect on the Wnt pathway in CBC cells, via a GPCR-dependent cascade to be identified, rather than a direct effect.

A more ambitious experiment that would help clarify the mechanism underlying the effects of Lgr4 in vivo would be to cross Lgr4 KO mice to mutant mice expressing active beta-catenin (Harada et al. EMBO J, 1999) and test if this rescues the loss of Paneth and stem cells. Being aware of the difficulties of such an experiment, though, I would not consider it indispensable for publication. Although less crucial in the context of a permissive role of Lgr4, we agree that this experiment is worth doing. We have asked permission to use the mice expressing active beta-catenin.

Unfortunately, the time allotted to the present revision will not allow inclusion of these experiments.

3) Since Lgr5 is a Wnt target gene itself, it could be interesting to test if Wnt modulation can also affect the levels of Lgr4.

Lgr4 has not been identified amongst the Wnt targets in the extensive screening of the Clevers group in which they identified 80 such targets (Van de Flier et al. Gastroenterology, 132, 628-632, 2007). In agreement with these data, we have measured the level of Lgr4 transcripts by qRT-PCR in Lgr5KO ileum at P0, in which we have shown that the Wnt pathway was activated (Garcia et al 2009), and found no significant modulation of Lgr4 expression in this context.

Specific Remarks:

a) The authors are invited to provide a bigger magnification for Fig.1A, since Lgr4 expression in the intervillus pockets of newborn mice is not obvious in the present picture.

We present a new version of figure 1 in which we have also added data from the developing intestine of E15 mice and larger panels for WT tissues.

b) In Fig.1E, the villi should be shown as well, in order to appreciate the crypt-restricted expression of Lgr4.

We have modified fig.1 to better illustrate the specific presence of transcripts in crypts and not in villi (new picture in the new Fig.1B).

c) The number of BrdU positive cells in Fig.2C should be normalized to the total number of crypt cells; since Fig.2A and B clearly show a reduced number of cells per crypt, this would lead to a reduced number of BrdU+ cells per crypt even if Lgr4 had no effect on crypt proliferation.

We have performed the normalization “per crypt cell” as suggested by the referee, and provide the results hereunder (see annex 3 to this letter). As predicted by the referee, the decrease of BrdU incorporation is less apparent but remains statistically significant at P15. We believe, however, that expressing the results “per crypt” is more meaningful, as it reflects the proliferative capacity of the CBC+TA unit and does not introduce the bias of non-proliferating Paneth cells (present in WT but not in KO). Our results suggest that in Lgr4KOs, in vivo, this proliferative capacity is reduced which explains the decrease in the number of cells per crypt and, secondarily, of crypt depth.

d) The size of the scale bars in Fig.3 and 4 is missing and should be included in the Figure Legends.

This has been corrected.
e) The authors are invited to provide a qRT-PCR or better a Northern blot showing the 10% residual expression of Lgr4 in the KO mice. About 10% of Lgr4 transcripts are indeed present in “Lgr4KOs”, by qRT-PCR. These transcripts are wild-type, corresponding to the splicing-out of the genetrap insert from some of the transcripts together with intron1. It implies inefficient termination of transcription at the polyA addition site of the construct. Data illustrating partial skipping of the cassette (by RT-PCR) and the residual level of WT transcript (by qRT-PCR) have been added and are presented in supplementary Fig S2A.

f) It is unclear if Lgr4 expression as well as its phenotypes are exclusively observed in the ileum or are found in other regions of the small intestine and the colon. X-gal staining on the different intestinal tracts of the Genetrap LacZ knock-in allele is needed, along with an evaluation of the reduction of Paneth cells and proliferating cells in the other regions of the small intestine of Lgr4 +/- mice. Studies of Lgr4 expression in heterozygotes (via X-gal staining) in the duodenum and colon, at P0 and in adults, are now added in Supplementary Fig. S1A. A same pattern of expression is observed in the proximal and distal small intestine while in the colon, the ¾ bottom of the crypts are positively labeled together with mesenchymal and smooth muscle cells. Concerning the effect of Lgr4 deficiency on the duodenum, data are now added in Supplementary Fig. S2D. Paneth cell deficiency is also observed in Lgr4KO mice while BrdU incorporation per crypt appears significantly decreased in Lgr4KO mice at P21 only.

g) The authors find by microarray analysis that, with the exception of Paneth cell specific genes, markers of terminal lineage differentiation are upregulated in Lgr4 KO organoids. It is not shown, though, if this reflects an increase in the number of differentiated cells in the "minigut" cultures coming from KO mice. Staining for those specific markers in the organoids from KO and WT mice would clarify this point. Our interpretation of this observation is that absence of self-renewal of CBCs in Lgr4KO organoids leads to non renewal of TA cells, with subsequent increase in the proportion of terminally differentiated enterocytes, Goblet and enteroeendocrine cells. In agreement with that hypothesis, we have performed immunofluorescence experiments on WT and Lgr4KO crypts at day 1. At this stage, “organoids” are spherical structures with a wall of stretched cells due to internal pressure (see figure 3). These are extremely fragile and difficult to study morphologically but we could observe a clear decrease in Ki67 nuclear labeling in Lgr4KO structures compared to WT (see new Supplementary Fig. S3C).

Regarding the query of referee to document differentiation markers in organoids, at day1, most of the spherical structures come from sealed crypts known to contain mostly stem cells and progenitors and only rare terminally differentiated cells except Paneth cells (in WT structures). Therefore, the chance to get a section with an enteroendocrine or a goblet cell is very low (verified experimentally). At any rate, qRT-PCR data presented in the new Fig. 3F and in Fig. 4C suggest that the relative increase in differentiated markers reflects the loss of stem cells in the cultures.

We hope that the new version will be found acceptable.

Sincerely yours
Annex 1.

Schematic representation of the functional relationships between CBCs, Paneth cells and mesenchyme, in WT and Lgr4KO crypts, in vivo and ex vivo, as modeled from our data. In WT crypts, in vivo, CBCs are maintained by Wnt signals coming from adjacent Paneth cells (Sato et al., 2010). Both Lgr4 and an undefined signal from the mesenchyme play a permissive role on Wnt signaling. In Lgr4KO crypts (∥ symbols), in vivo, the Wnt pathway is functional in CBCs thanks to the complementing effect of the mesenchymal factor. Lack of Lgr4 in CBCs and possibly in early Paneth cells would interfere with terminal differentiation of Paneth cell. Complementation by the mesenchymal factor would be ineffective in early Paneth cells, because they lack the corresponding receptor. In Lgr4KO crypts ex vivo (∥ + \ symbols), the effect of both Lgr4 and the mesenchymal factor deficiency would render Wnt signaling inefficient, despite continuing production of Wnt3 and Wnt11 by Paneth cell precursors: the crypts die from absence of cell renewal. Addition of LiCl to the culture medium partially compensates for the lack of Lgr4 and the mesenchymal factor.
Annex 3

Quantification of BrdU staining with normalization to total number of cells in crypts (20 crypts per mouse; n=2-7 per group);
* p<0.025;  t p<0.05.

2nd Editorial Decision 10 March 2011

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. While both referees 1 and 3 support now publication of the study in EMBO reports, referee 2 does not. After additional discussion with both referees 1 and 3 we came to the decision that you should address referee 2’s concerns a bit more explicitly in the manuscript text before we can proceed with the official acceptance of your manuscript.

I also noticed that the figure legends for figures 2, 3, and 4 do not state the test used to calculate the p-values and do not always define the error bars. Please include the missing information in the figure legends.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports
REFEREE REPORTS

Referee #1 (Remarks to the Author):

The Manuscript entitled "Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells" has been improved by Mustata et al. In particular, they clearly show no effect of wnt3a addition at concentrations that should bypass the need of Paneth cells for organoid growth from Lgr4KO crypts. In addition, they demonstrate that while crypts in culture lack terminally differentiated Paneth cells, precursors present at day 0 of organoid culture are able to provide growth signals to CBCs. These two experiments rule out that lack of organoid growth from Lgr4KO crypts could be due to deficient Paneth cells. Authors have rephrased the text to accommodate the notion of a permissive role of Lgr4 for Wnt signaling rather than an activating role, which is compatible with their results, and clearly state that compensatory signals exist in vivo to overcome Lgr4 deficiency. The authors have addressed the main concerns of this referee.

Referee #2 (Remarks to the Author):

The manuscript and the figures have been improved. The study focuses on the role of Lgr4 in the intestinal epithelium and reports and describes the impact of Lgr4-deficiency both in vivo and ex vivo. One of the main conclusions of the study is that Lgr4 provides permissivity to Wnt signals in CBC stem cells. However, data that strongly support this view are still lacking.

1- The authors's statement "Our observations, in vivo, point to a role of Lgr4 in keeping Wnt stimulatory tone at a level compatible with normal crypt development" is not in agreement with figure 3E, which shows identical levels of expression of the tested Wnt target genes (Lgr5, Ascl2, Axin2, Sox9, etc) in P15 Lgr4KO and WT mice, at the time of plating.
2- Although Lgr4-deficient organoids seeded from P15 mice fail to grow, this phenotype is not rescued by adding Wnt ligands and is only very partially rescued by LiCl, but not by Gsk3 inhibitors, suggesting that the LiCl-mediated rescue may well be independent of Wnt signalling. The authors suggest such a possibility but do not take it in account to raise their conclusions. Therefore, and although I agree with the authors that a detailed mechanistic study is beyond the scope of this manuscript, I cannot but disagree with their conclusion that Lgr4 provides permissivity to Wnt signals.

Referee #3 (Remarks to the Author):

The revised manuscript is improved. Data presented are convincing. Presentation of the manuscript is clear, discussion is adequate. I recommend publication of this work as it stands.

We thank you and reviewers 1 and 3 for a positive appreciation of the revised version of our manuscript.

We have considered the two points of referee 2 and modified our text in a way which, we hope, will meet your expectations.

Regarding the question about the statistics in figs 2, 3 and 4, we have completed the legends as requested.
We hope our manuscript will now be found acceptable.

Yours sincerely

Point #1.

The authors's statement "Our observations, in vivo, point to a role of Lgr4 in keeping Wnt stimulatory tone at a level compatible with normal crypt development" is not in agreement with figure 3E, which shows identical levels of expression of the tested Wnt target genes (Lgr5, Ascl2, Axin2, Sox9, etc) in P15 Lgr4KO and WT mice, at the time of plating.

We agree that this sentence may sound contradictory with data in Figure 3E. However, we think that a minimal effect of Lgr4KO in vivo, affecting proliferation in the crypts, is not incompatible with WT levels of transcripts of Wnt target genes. Indeed, the relative proportion of CBCs (and of their marker transcripts) in the smaller Lgr4KO crypts may not be significantly affected. We have nevertheless modified the sentence by making it simply descriptive, leaving the interpretation for the conclusion sentence later in the paragraph. This conclusion takes the form of a hypothesis which, we think, is logical given the known effects of Wnt on intestinal crypts. We have slightly modified this conclusion sentence by adding reference to the role of Wnt signaling on Paneth cell differentiation.

Alterations introduced in the text are underlined (see below)

Point #2.

Although Lgr4-deficient organoids seeded from P15 mice fail to grow, this phenotype is not rescued by adding Wnt ligands and is only very partially rescued by LiCl, but not by Gsk3 inhibitors, suggesting that the LiCl-mediated rescue may well be independent of Wnt signalling. The authors suggest such a possibility but do not take it in account to raise their conclusions. Therefore, and although I agree with the authors that a detailed mechanistic study is beyond the scope of this manuscript, I cannot but disagree with their conclusion that Lgr4 provides permissivity to Wnt signals.

In the absence of a precise biochemical/mechanistic explanation, we used the word "permissive" in a purely descriptive way for both Lgr4 and LiCl effects. Since Lgr4KO organoids die ex vivo despite adequate presence of endogenous Wnt ligands (as it is likely, from our qRT-PCR data), or addition of exogenous Wnt3a, it means that presence of Lgr4 is required for Wnt signaling to be effective, which is what we meant by "permissive". A similar reasoning applies for the partial LiCl effect.

We have modified the penultimate paragraph to clarify our view and removed the word "positively" in the penultimate sentence of the last paragraph to account for the fact that our conclusions are reached essentially from loss of function studies.

Here are the last two paragraphs of the manuscript, with the alterations indicated.

In line with almost normal levels of Wnt transcripts in Lgr4KO crypts at day 0, the absence of rescuing effects of exogenous Wnt3a on Lgr4KO crypts suggests that death of CBC cells or P0 progenitors is cell-autonomous, with Lgr4 acting as a survival factor, most likely via a permissive effect on the Wnt signaling cascade. Precise dissection of the connections between Lgr4 and Wnt regulatory pathways will only be possible when Lgr4 agonist(s) will become available. Failure of gsk3β inhibitors to rescue Lgr4KO crypts ex vivo suggests that LiCl acts, at least partially, independently of its known inhibitory effect on this enzyme (Phiel and Klein, 2001). In this regard, LiCl has recently been shown to control gsk3β by dissociating βarrestin-PP2a-Akt complexes (Beaulieu et al., 2009). This provides an interesting link with GPCRs and suggests that the action of LiCl (and, maybe Lgr4) could involve interaction between the Wnt and PI3K/Akt pathways.
Our observations, in vivo, point to a role of Lgr4 in allowing normal cell proliferation in intestinal crypts and terminal differentiation of Paneth cells. Ex vivo, they demonstrate an absolute requirement of Lgr4 for the maintenance of crypt stem cells, which contrasts with the survival and development into organoids of Lgr5KO progenitors. The milder phenotype of Lgr4KO observed in vivo implies that extra-epithelial signals of systemic or, more likely, mesenchymal or muscular origin, (Powell et al., 2011) partially compensate for Lgr4 deficiency in vivo. Given the documented role of Wnt on stem cell survival and proliferation, as well as on Paneth cell differentiation, we hypothesize that Lgr4 positively controls the Wnt pathway in CBCs in a permissive way. Our results demonstrate non redundancy between Lgr4 and Lgr5 genes and identify Lgr4 as a potential target for the development of antagonists with therapeutic use in intestinal cancers.

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Yours sincerely,

Editor
EMBO Reports