Mapping early fate determination in Lgr5+ crypt stem cells using a novel Ki67-RFP allele

Onur Basak, Maaike van de Born, Jeroen Korving, Joep Beumer, Stefan van der Elst, Johan H. van Es and Hans Clevers

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 19 February 2014

Thank you very much for the opportunity to consider your results addressing the cell cycle status of intestinal stem cell populations employing a novel in-vivo reporter for potential publication in The EMBO Journal.

Two expert scientists commented on the presented results. While offering some support, at least the very thorough and constructive assessment from referee #1 surfaces significant caveats that would demand extensive further experimentation before him/her becoming fully convinced and thus supportive of the major conclusions. Specifically, functional isolation/interrogation of Lgr5-negative cells in distinct cell cycle stages would, according to this scientist, reveal a much more complete and convincing picture. While apparently technically rather challenging, some progress/advance in this direction would be much appreciated as to raise the overall profile and significance of the study. Together with a broader characterization of the cellular lineages under investigation as well as overcoming current internal contradictory elements, I would be pleased to approach ref#1 with an appropriately revised/amended version.

Please notice at this point that The EMBO Journal considers only one round of revisions and we would be prepared to discuss about necessary extension of our three-month deadline given the relatively broad experimental demands.
Please do not hesitate to get in touch to outline feasibility/planned experiments/realistic timeline to address the referee critiques. On such a basis, I am very much looking forward to hear from you and remain with best regards.

REFEREE REPORTS:

Referee #1:

The manuscript by Basak et al aims to resolve the cell cycle status of intestinal stem cells using a novel reporter mouse in which a red fluorescent reporter gene is knocked into the Ki67 locus. Ki67 is a widely used indicator of cell cycle status that is commonly detected in situ by immunohistochemistry. It is expressed in all stages of the cell cycle G1/S/G2/M but not in G0 cells. The reporter mouse offers the potential to isolate and functionally characterize cells based on their cell cycle status.

The authors apply this reporter in combination with their widely used Lgr5GFP reporter knockin line, assess the cell cycle status of the Lgr5 positive populations and functionally characterize and test them. This forms the latest initiative of the group to provide a consensus view of the different stem cell marker expression profiles and properties of crypt base cells. Overall their findings appear consistent with a model in which loss or diminishment of Lgr5 expression is coincident with commitment and with continued Ki67 positivity broadly associating with absorptive lineages that require many additional amplifying divisions. Loss/reduction of Lgr5 without Ki67 positivity associates with commitment to secretory lineages.

The objective is worthwhile, the approach taken is novel and the results interesting. There are significant caveats. By defining a rather narrow term of reference it does appear that the authors have missed an opportunity. The Ki67/RFP approach could potentially have been applied to test crypt base populations without regard to Lgr5 status at least in the first instance. Indeed it is striking that the one population that remains untested in the study are the Lgr5 negative cells in different cell cycle states. It is understood that this is due to the mosaic nature of the Lgr5 mouse that means GFP cells may express Lgr5 but have the authors not yet got access to an Lgr5GFP mouse that lacks such mosaicism?

A more 'blinded' approach might have helped also with the absence of actual evidence for Lgr5GFPlow/Ki67+ cells reflecting enterocyte lineages with their subsequent transit amplifying divisions. Fig7 purported to show this (page 12 and legend) but this poorly annotated figure (panels are not described) deals only with secretory markers. Further there seems an under-representation of proliferation associated genes in Fig4A meaning that we never convincingly see Ki67/RFP acting as a cell cycle indicator.

There are clear contradictions in elements of the presentation. The observation that Lgr5Hi/RFP+ and Lgr5Hi/RFP- cells are essentially transcriptionally identical is interpreted as supporting the case that all Lgr5Hi cells are actively cycling. If this is accepted then RFP negativity (12% of the Lgr5Hi population) again cannot be trusted as an indicator of cell cycle status. More validation of the reporter is required.

Relatedly, the cells derived from the Ki67-RFP knockin mice show that RFP+ and not RFP- cells can form organoids with the authors interpreting this as evidence that all stem cell activity resides in the cycling population. How is this compatible with the observation that Lgr5Hi/Ki67- cells are equivalent to Lgr5Hi/Ki67+ cells in clonogenic potential?

From Fig2 the Ki67RFP seems to be expressed in most cells of the crypt but in the FACS plot shown only 45.6% of cells are gated as RFP+. It seems possible from this analysis that the crypt cell isolation method gives both crypt and significant villus cell contamination. FACS analysis with an additional crypt marker might address this.

The RFP signal seems relatively weak: an alternative explanation might be that there is some portion of the cell cycle where the RFP drops below detection threshold. In this regard some quantification of the claimed absolute correlation between immuno fluorescence for Ki67 and the RFP in Figure 2...
would be appropriate. Also co-staining with a DNA binding dye and showing the DNA content of the RFP+ population would reassure that there is no bias across the cell cycle.

DLL1+ and LRCs generate organoids yet Lgr5Low/Ki67- do not. This would not appear to support functional equivalence as implied.

Minor Points
Is the single cell organoid culture as inefficient as suggested by a 1% growth of Lgr5Hi/Ki67+ cells? If only 10 of the 1000 plated actually grow the dynamic range for this experiment is low.

Fig 1B is not adequately explained in results with respect to the significance of Lysozyme positivity.

Fig 1D labelling needs addressing: the column showing the distribution of chromogranin positive cells (column 3 of last panel) are labelled as negative for ChgA.

The datasets comparisons of in Fig 4D and accompanying text are unclear. Heijmans et al 2013 is quoted in text as the source of the stem cell signature genes and that paper in turn refers to van der Fier et al 2009, while the figure legend refers to Munoz et al 2012. Are the 200 top stem cell genes basically from Lgr5Hi cells? In which case are the authors comparing to their own data for the same cells?

In Fig 4B , second panel for Ki67- cells there seems to be group of outliers that fall underneath the main cloud of linearly distributed transcripts that are absent in the first panel (Ki67+) cells. What genes are represented here?

Referee #2:

Ever since Lgr5 was discovered as CBC stem cell marker in the intestine there has been great interest in the nature of these cells, their dispensability and the overlap with previously reported quiescent stem cells which resided at position 4. Over the past few years now a plethora of stem cell markers at different positions have been discovered including LRIG1, BMI1, HOXP. This manuscript will be an important addition to the field where a consensus now appears to be emerging. Elegant work by the Clevers laboratory on DLL1 and the Winton laboratory on LRC (label retaining cells) suggest a back up slowly cycling secretory-like cells which may repopulate the crypt following injury and the loss of Lgr5 cells.

In this elegant paper the Clevers laboratory now use the proliferative capacity of the cells (via a Ki67 RFP knock-in mice) to identify whether these populations exist in the crypt and their inter-relationship with Lgr5+ Cells. They show clearly that there are 2 populations of Lgr5+ cells both Ki67+ and negative which are similar, colony forming but do not constitute these back up cells. Whilst the Ki67- Lgr5- at the crypt base resemble those of Winton and appear the backup progenitors. Interestingly these are not colony forming in vitro.

Overall the data within the manuscript is very clear and builds on some excellent studies already published. I believe given all the controversy over the LRC population and Lgr5 this is still a very valuable story building on these previous ones.

Minor comments.
1. Figure 1 is mislabelled. 1D second panel I assume it should be Lgr5 on bottom axis not CHGA+
2. Given cells at the top of the crypt would like be Lgr5 negative and Ki67 negative, the authors should given more details in their methods on how they ensured these were not included in their analysis.
3. Some discussion of the finding that in figure 1 Ki67 negative cells were not colony forming whilst in the next section Lgr5+ Ki67 negative cells could colony form. I assume this is just because of the enrichment for Lgr5 cells and in the whole crypt Ki67 negative cells would be from a much larger set of cells.
4. Given the secretory population repopulates the intestine following injury this would argue the
The objective is worthwhile, the approach taken is novel and the results interesting. There are significant caveats. By defining a rather narrow term of reference it does appear that the authors have missed an opportunity. The Ki67/RFP approach could potentially have been applied to test crypt base populations without regard to Lgr5 status at least in the first instance.

A: We thank the reviewer for his appreciation of our new mouse model, experimental approach and results. Indeed, Ki67<sup>RFP</sup> mouse can be used to analyse the progenitor features independent of additional marker and is a very plausible way of validating our reporter. However, we would like to
note that such an approach has a downfall: Differentiated crypt cells will also be in the RFP-fraction.

To address the reviewers point, we have sorted RFP+ and RFP- populations from the intestinal crypts of the Ki67\^RFP mice. First, we analysed the differences in their gene expression pattern using quantitative PCR. As expected, proliferation markers Ki67 (27.9 fold; 19.2 ± 7.1 vs 0.7 ± 1.0; p=0.043) and Ccnb2 (3.5 fold; 3.6 ± 1.8 vs 1.0 ± 1.1; p=0.003) were highly enriched in Ki67+ population. The Paneth cell marker Lyz (2.6 fold; 0.3 ± 0.4 vs 0.8 ± 1.5; p=0.003) was not significantly different between Ki67+ and – cells, and was below the crypt average in both populations. The enterocyte/secretory progenitor marker ChgA (25.9 fold; 0.2 ± 0.1 vs 4.1 ± 1.6; p=0.048) and the absorptive Enterocyte marker Alpi (2.3 fold; 0.3 ± 0.3 vs 0.8 ± 0.3; p=0.035) were enriched in the Ki67- fraction. Consistent with our previous results and the results shown here, we didn’t find any difference in the ‘+4’ marker Bmi1 (1.2 fold; 0.9 ± 0.5 vs 1.0 ± 1.0; p=0.842; Data is added only to the text). We added the data in the new panel Figure 2G and mentioned in the text on page 6.

Indeed it is striking that the one population that remains untested in the study are the Lgr5-negative cells in different cell cycle states. It is understood that this is due to the mosaic nature of the Lgr5 mouse that means that GFP- cells may express Lgr5 but have the authors not yet got access to an Lgr5GFP mouse that lacks such mosaicism?

A: As the reviewer mentioned, the reason behind a lack of analysis of the Lgr5 negative crypt cells was the mosaic expression of the Lgr5GFP allele by a fraction of Lgr5+ cells. To circumvent this, as suggested by the reviewer, we have generated Lgr5\^GFPDTR\^Ki67\^RFP mice, where every Lgr5+ cell expresses GFP. However, sorting GFP expressing populations has the unique advantage of excluding differentiated crypt cells and any possible contamination from the crypt-villus junction. Thus by analysing the Lgr5-GFP negative populations we unfortunately cannot exclude the differentiated cells in the Ki67- compartment. Nevertheless, we performed details analysis of the Lgr5- crypt populations.

We isolated Lgr5\^Ki67+ (L-K+) and Lgr5\^Ki67- (L-K-) populations by FACS and analysed the differences in their gene expression pattern using quantitative PCR (n animals=3). Lgr5 mRNA expression was constantly enriched in Lgr5-GFPDTR sorted cells on average by 32 fold (28.7±14.1 vs 0.9±0.3). As expected, proliferation markers Ki67 (16.8±13.4) and Ccnb2 (2.50±0.85) were highly enriched in L-K+ compared to L-K- population. Transcription factors regulating secretory differentiation, Atoh1 (0.04±0.04) and NeuroD1 (0.01±0.00) were exclusively expressed in L-K- cells, consistent with an inverse correlation between secretory differentiation and cell cycle progression. Paneth cell marker Lysozyme (Lyz; 0.29±0.15), the Goblet cell marker Gob5 (0.09±0.04) and the enteroendocrine/secretory progenitor marker ChromagraninA (ChgA; 0.01±0.00) were similarly enriched in non-dividing Lgr5- crypt fraction. We observed an enrichment of Fabp2 (0.46±0.01) and Alpi (0.44±0.12) in L-K- cells compared to the L-K+ population. Both of these genes are up-regulated in upon enterocyte differentiation and are expressed at lower levels in the crypt. Finally, even though we cannot exclude a rare quiescent population with high levels of Bmi1 expression, we failed to detect a striking correlation between cell cycle progression and Bmi1 expression, which is slightly higher in L-K- cells (0.59±0.02). These results are presented in Supplementary Figure 3 and mentioned in the text as a new section on pages 11-12.

A: We agree that the assumption that the Ki67+ cells are absorptive lineage is not well supported.
with the data presented. Analysis of absorptive lineage markers Alpi and Fabp2 in crypt populations isolated from Ki67\textsuperscript{RFP} and Lgr5\textsuperscript{GFPDTR}\textsuperscript{Hi}Ki67\textsuperscript{RFP} mice using qPCR suggest a slightly higher expression of the absorptive lineage markers in Ki67- population. However, interpretation of these results requires caution. We cannot exclude that few post-mitotic Enterocytes are among the Ki67\textsuperscript{RFP} cells, which due to low expression of these markers by crypt populations might influence our results. In the absence of evidence, we have withdrawn the claim from the text and the model and left it as a speculation in the discussion.

Further there seems an under-representation of proliferation associated genes in Fig4A meaning that we never convincingly see Ki67/RFP acting as a cell cycle indicator.

A: Point is taken. We now show that Ki67\textsuperscript{RFP} expression correlates with the cell cycle using 2 different approaches. First, we performed HOECHST-34580 staining on dissociated live intestinal crypt cells (n=3). HOECHST dyes bind DNA and directly measures the amount of DNA present in a cell. Since both cycling G\textsubscript{1} phase and non-cycling G\textsubscript{0} phase cells have one set of chromosomes, we can visualize a peak (G\textsubscript{0}/1) in both cases. Active DNA synthesis results in higher DNA content and HOECHST incorporation, labelling cells in the S, G\textsubscript{2} and M phases of the cell cycle (S-M). On average 12.3 % (±3.3) of the crypt cells were in S-M phases of the cell cycle. 52.9% (±9.8) of the Ki67\textsuperscript{RFP}+ cells were in S-M phase of the cell cycle, confirming that RFP expression correlates with cell cycle progression. In sharp contrast, only 4.8% (±2.5) of the Ki67\textsuperscript{RFP}- cells is in S-M phases of the cell cycle indicating a lack of cell cycle activity. Of note, the small fraction of Ki67\textsuperscript{RFP}- cells had only slightly higher HOECHST signal than the G\textsubscript{0}/1 peak. A plausible explanation is that Ki67\textsuperscript{RFP} might not yet be expressed above our detection level in the early S-phase. Alternatively, this could be a mere result of a lack a sharp G1 peak using the HOECHST. These are presented in new Figure 2E, F and added in the text on page 6.

Secondly, as mentioned above, we used qPCR to show that the expression of Ki67 (27.9 fold; 19.2 ± 7.1 vs 0.7 ± 1.0; p=0.043) and Ccnb2 (3.5 fold; 3.6 ± 1.8 vs 1.0 ± 1.1; p=0.003) are highly enriched in Ki67+ fraction compared to the Ki67- fraction, confirming Ki67\textsuperscript{RFP} as a marker of proliferating cells. These results are shown in Figure 2G.

There are clear contradictions in elements of the presentation. The observation that Lgr5\textsuperscript{Hi}/RFP+ and Lgr5\textsuperscript{Hi}/RFP- cells are essentially transcriptionally identical is interpreted as supporting the case that all Lgr5\textsuperscript{Hi} cells are actively cycling. If this is accepted then RFP negativity (12% of the Lgr5\textsuperscript{Hi} population) again cannot be trusted as an indicator of cell cycle status. More validation of the reporter is required.

A: We understand the concern about the lack of cell cycle indicators in Lgr5\textsuperscript{Hi}Ki67- population. We have validated the reporter better with the experiments mentioned above. Ki67\textsuperscript{RFP} expressing cells express high levels of cell-cycle specific genes and most are in S-M phases of the cell cycle, implicating them as proliferating cells.

We also show that some of the RFP+ cells express the Ki67 protein, even though RFP- cells are not in S-M phases of the cell cycle. Thus, the lack of RFP expression in the Lgr5\textsuperscript{Hi} population but the presence of the mKi67 RNA could reflect the early G1 phase where Ki67 is transiently absent or below our detection threshold. Thus we interpreted our results, as an indication that the Ki67- state of Lgr5\textsuperscript{Hi} cells is transient, being followed by cell cycle re-entry or loss of Lgr5 expression and thus exit from the Lgr5\textsuperscript{Hi} Ki67- state. As Lgr5 levels drop, the differences between Ki67+ and Ki67- states become apparent. Results are presented in the text on page 6 and in the discussion.

Relatedly, the cells derived from the Ki67-RFP knockin mice show that RFP+ and not RFP- cells can form organoids with the authors interpreting this as evidence that all stem cell activity resides in the cycling population. How is this compatible with the observation that Lgr5\textsuperscript{Hi}/Ki67- cells are
equivalent to Lgr5Hi/Ki67+ cells in clonogenic potential?

A: Point taken. We can roughly calculate that the Lgr5highKi67- cells form only a fraction of the Ki67- population 0.3/50% = 0.6%. We likely did not capture these cells in our analysis. We now repeated the experiments using a higher number of cells from both Ki67+ (0.43±0.28%) and Ki67- (0.05±0.04%) fraction using higher number of cells. We show that despite being much less than the Ki67+ fraction (0.43±0.28%), Ki67- cells can form organoids (0.05±0.04%). These results are added in the text on page 6-7 and Figure 2 J, K.

From Fig2 the Ki67RFP seems to be expressed in most cells of the crypt but in the FACS plot shown only 45.6% of cells are gated as RFP+. It seems possible from this analysis that the crypt cell isolation method gives both crypt and significant villus cell contamination. FACS analysis with an additional crypt marker might address this.

A: We agree that the percentage of Ki67+ cells could be higher than 45% within the crypt. However, as all our sorts are performed using Lgr5GFP expression, villus cells definitely did not affect our analysis of GFP expressing populations. In addition, as seen in Fig 3A, the majority of the cells in our preparation are crypt cells. Nevertheless, in our new sorts to isolate Lgr5DTR and Ki67 populations we addressed this question using qPCR on isolated crypt and villus populations. We show a clear enrichment of Ki67 (97.5x), Cnb2 (12.5x), Lgr5 (30x), Chga (2.5x) and Lyz (82x) in the crypt fraction, while Alpi (2.3x; p=0.016) and Fabp2 (3.7x; p=0.044) are significantly more in the villus fraction. Even though some villus contamination in our preparations can be anticipated, the majority of our preparation is composed of crypt cells. These results are in Supplementary Figure 1 and in the text on page 6.

The RFP signal seems relatively weak: an alternative explanation might be that there is some portion of the cell cycle where the RFP drops below detection threshold. In this regard some quantification of the claimed absolute correlation between immuno fluorescence for Ki67 and the RFP in Figure 2 would be appropriate.

A: We agree that some RFP+ cells might be mistaken as RFP- due to technical reasons related to the available FACS setup and lack of an optimal laser for TagRFP excitation. We now quantified crypt cells sorted for Ki67RFP expression using the same gate used throughout the study and stained using anti-Ki67 antibody. 96.6% (±5.6%) of RFP+ sorted cell were Ki67+, while 14.4% (±7.6%) of the RFP- cells expressed the Ki67 protein. These results suggest that indeed a population of Ki67+ cells fall below our threshold. This could either be a result of low Ki67 expression, or a gap during the maturation period of the TagRFP protein. These results are incorporated into the text on page 6 and Figure 2I, and discussed in the discussion.

Also co-staining with a DNA binding dye and showing the DNA content of the RFP+ population would reassure that the there is no bias across the cell cycle.

A: As mentioned above, we performed HOECHST staining on dissociated intestinal crypts and show that RFP expressing cells are highly likely to be in the S-M phases of the cell cycle (Figure 2E, F, page 6). It is very hard to judge a bias in specific cell cycle phase (e.g. more in M than G1) using this method, however, we can confirm that Ki67RFP is expressed by cells in both G1 and S-M phases.

Dll1+ and LRCs generate organoids yet Lgr5low/Ki67- do not. This would not appear to support functional equivalence as implied.

A: We agree that there is a clear difference in the ability of Dll1+ and Lgr5low/Ki67- cells to form
organoids. Dll1+ cells have slow cell cycle dynamics but expand to generate multiple cells, indicating proliferative potential. It is likely that using the Ki67-RFP allele we capture a fraction of Dll1 cell that are out of the cell cycle. We corrected the model figure and discussed in the text on page 14 to stress the overlap, rather than functional equivalence, of Dll1+ and Lgr5\textsuperscript{low}Ki67+ populations.

Minor Points

Is the single cell organoid culture as inefficient as suggested by a 1% growth of Lgr5\textsuperscript{Hi}/Ki67+ cells? If only 10 of the 1000 plated actually grow the dynamic range for this experiment is low.

A: Due to technical reason (detection of RFP), we have chosen to use BD FacsAria to perform the sorts. Unfortunately, we repeatedly obtain low efficiency of organoid initiation in cultures started from cells sorted from the BD Aria, in contrast to the MoFlo that we have used in our previous studies. Due to the low dynamic range, we might have missed some rare organoid forming cells in our Lgr5\textsuperscript{low}Ki67+ population, however, we show a clear relative inability to form organoid compared to other Lgr5 populations cultured from same animals in parallel.

Fig1B is not adequately explained in results with respect to the significance of Lysozyme positivity.

A: Lysozyme staining is used to determine the Paneth cell zone. We added a sentence in the text on page 6 and changed the figure legend.

Fig1 D labelling needs addressing: the column showing the distribution of chromogranin positive cells (column 3 of last panel) are labelled as negative for ChgA.

A: We apologise for the mistake happened during figure preparation. We have now corrected Fig 1D.

The datasets comparisons of in Fig4D and accompanying text are unclear. Heijmans et al 2013 is quoted in text as the source of the stem cell signature genes and that paper in turn refers to van der Fier et al 2009, while the figure legend refers to Munoz et al 2012. Are the 200 top stem cell genes basically from Lgr5\textsuperscript{Hi} cells? In which case are the authors comparing to their own data for the same cells?

A: We thank the reviewer for correcting the confusion in citations. Heijmans et al cites van der Flier et al for the geneset they have used in their GSEA analysis, however, uses the data set from Munoz et al 2012. The array analyses were performed by the group using the Lgr5-GFPiresCreER mouse (the same one used in this study) with 5 different expression levels, which we used to validate that our Lgr5-GFP Ki67-TagRFP double sort paradigm worked successfully to distinguish stem cells from their progeny. We have now excluded Heijmans et al 2013 from the figure legends. We added a sentence stressing that the analysis is done to confirm our sorting paradigm using the published Lgr5-GFP signature. We also corrected the citation the source of the gene list for Paneth, enteroendocrine and Dll1+ cells from Sato et al to van Es et al and added the citations in the figure legends for figure 6 (page 28) and materials and methods (page 19).

In Fig4B, second panel for Ki67- cells there seems to be group of outliers that fall underneath the main cloud of linearly distributed transcripts that are absent in the first panel (Ki67+) cells. What genes are represented here?
A: We apologize for the confusion. These are the genes that displayed a very high variation between samples. As a result, even though the averages were over 2-fold enriched, they were not significantly different between samples. We have excluded these genes from the figure.

Referee #2:

Ever since Lgr5 was discovered as CBC stem cell marker in the intestine there has been great interest in the nature of these cells, their dispensability and the overlap with previously reported quiescent stem cells which resided at position 4. Over the past few years now a plethora of stem cell markers at different positions have been discovered including LRIG1, BMI1, HOXP. This manuscript will be an important addition to the field where a consensus now appears to be emerging. Elegant work by the Clevers laboratory on DLL1 and the Winton laboratory on LRC (label retaining cells) suggest a back up slowly cycling secretory-like cells which may repopulate the crypt following injury and the loss of Lgr5 cells.

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Overall the data within the manuscript is very clear and builds on some excellent studies already published. I believe given all the controversy over the LRC population and Lgr5 this is still a very valuable story building on these previous ones.

Minor comments.

Figure 1 is mislabelled. 1D second panel I assume it should be Lgr5 on bottom axis not CHGA+

A: We apologize for the mistake happened during figure preparation. We have now corrected Fig 1D.

Given cells at the top of the crypt would like be Lgr5 negative and Ki67 negative, the authors should given more details in their methods on how they ensured these were not included in their analysis.

A: One advantage of using Lgr5-GFP + (both high and low) cell populations is the exclusion of such Lgr5- cells at the top of the crypt from our analysis. These populations, however, influence our results in the analysis of Ki67-RFP populations in figure 2 and the newly added Lgr5- Ki67 populations in supplementary figure 3. To clarify the populations included in the study, we used qPCR on isolated crypt and villus populations. We show a clear enrichment of Ki67 (97,5x), Ccnb2 (12,5x), Lgr5 (30x), ChgA (2,5x) and Lyz (82x) in the crypt fraction, while Alpi (2,3x; p=0.016) and Fabp2 (5,7x; p=0.044) are significantly more in the villus fraction. Thus, even though some villus contamination in our preparations can be anticipated, the majority of our preparation is composed of crypt cells. These results are in the new Supplementary Figure 1 and in the text on page 6.

Some discussion of the finding that in figure 1 Ki67 negative cells were not colony forming whilst in the next section Lgr5+ Ki67 negative cells could colony form. I assume this is just because of the enrichment for Lgr5 cells and in the whole crypt Ki67 negative cells would be from a much larger set of cells.

A: Point taken. We can roughly calculate that the Lgr5highKi67- cells form only a fraction of the
Ki67- population 0.3/50% = 0.6%. We likely did not capture these cells in our analysis. We now repeated the experiments using a higher number of cells from both Ki67+ and Ki67- fraction. And show that despite being much less than the Ki67+ fraction, Ki67- cells can form organoids. These results are added to the new figure 2 J, K and in the text on page 6-7.

Given the secretory population repopulates the intestine following injury this would argue the conditions developed in the organoid cultures cannot mimic this. Some discussion of this should be placed in the text.

A: We agree that a lack of organoid growth from secretory precursors that can regenerate the intestine suggests additional factors play a role in their activation. We discussed this point in the discussion on page 14.

In the final discussion the authors mention about potential pathways that may let these secretory cells repopulate the niche. Given the major differences they show in these progenitors is that they have low levels of Wnt signalling compared to control the most likely explanation is that Wnt is activated in these cells. This is further backed up by the findings DLL+ cells give rise tumours following Apc loss and a number of studies have shown that Wnt/MYC is activated in intestinal regeneration and that even villi can be dedifferentiated by activating Wnt and KRAS mutation. These should be discussed.

A: Indeed, Wnt, myc and ras signalling have been implicated in intestinal stem cell proliferation, regeneration and dedifferentiation of villus cells into undifferentiated crypt cells. These pathways might as well play a role in transition from a quiescent into an active form. We discussed these possibilities in the text on page 15.

Other changes:

Supplementary figure 1 is renamed as supplementary figure 2. Relevant changes were made in the text and the figure legends.

2 new supplementary figures are included (Supp Fig1 and Supp Fig3). Relevant citations to the figures are added in the text and new figure legends are included.

In figure 1D, at the bottom of the most right panel, label CHGA is changed into LGR5.

In Figure 2, Figure 2E is moved to 2H. Figure 2F is split and moved to 2J and 2K. Images in 2J are renewed, quantification in 2K is updated. New panels 2E, F, G and I are included.

In figure 5D, the grey coloured gene names are deleted.

The model on figure 7 is revised after the reviewers’ suggestions.
We have received the final referee report and I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal, pending minor amendments to the document file as to accommodate the enclosed referee remarks as you deem constructive and appropriate.

Please provide us with a final dock-file via reply E-mail to your earliest convenience.

I also kindly ask you at this point to provide a 2 up to 4 bullet-point synopsis that highlights the major findings of your study in a short and concise manner.

Please accept my congratulations to such a fine paper and excellent revisions to reach the approval of our referees.

REFEREE REPORT:

In this revised manuscript Basak and colleagues have done a very good job at addressing the comments of the referees. The inclusion of the LGR5-GFPDTR mouse is appreciated as this overcomes issues of mosaicism of the original LGR5-GFP-CREER mouse. Also appreciated is the more robust characterisation of the KI67-RFP mouse (its pleasing to see the overlap between Ki67-RFP and KI67 expression).

The authors have made the appropriate revisions to the text and have properly discussed points (and reduced over-interpretation of some points).

My only minor suggestions are:
1. that the authors should check their stats as it odd that 96.6% (+5.5%) cells were positive for RFP, can you have over 100% of cell labelled?!
2. The manuscript terminology can get a little bit confusing throughout: For example the authors discuss position of crypt +1 to +5 and then have a 5 point scale for Lgr5-GFP positivity. The authors should check for consistency and ensure that the manuscript is accessible for the general reader.

Comments:

My only minor suggestions are:
1. that the authors should check their stats as it odd that 96.6% (+5.5%) cells were positive for RFP, can you have over 100% of cell labelled?!
2. The manuscript terminology can get a little bit confusing throughout: For example the authors discuss position of crypt +1 to +5 and then have a 5 point scale for Lgr5-GFP positivity. The authors should check for consistency and ensure that the manuscript is accessible for the general reader.

A: This is likely because we cannot specify an upper limit of 100% while calculating the standard deviation. The percentage of KI67 positivity in 4 sorts for RFP+ cells were 100%, 97.96%, 100%, 88.34% resulting in an average of 96.55% with a standard deviation of 5.63. We confirmed that calculations are correctly done and kept the data in the text.

A: To prevent any confusion, we excluded the nomenclature used for GFP levels and modified the sentences on page 7 as follows:
“We have previously identified stem cells and their progeny using GFP expression from the Lgr5 locus (Munoz et al, 2012). Here we focused on stem cells (Lgr5high) and their immediate progeny (Lgr5low) and we sorted KI67+ and KI67- subpopulations.”