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Atoh1^{+} secretory progenitors possess renewal capacity independent of Lgr5^{+} cells during colonic regeneration

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

1st Editorial Decision 10th Jul 2018

Thank you for the submission of your manuscript (EMBOJ-2018-99984) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

The referees acknowledge the potential interest and relevance of your work, although they also express major concerns, which would need to be conclusively addressed before they can be supportive of publication in The EMBO Journal. Referee #1 raises issues regarding the novelty of your findings in light of recent work showing contribution of Atoh1+ cells to colonic regeneration post injury. Referee #2 states that your claims on Lgr5-independent activity of the Atoh1-cells and their in vivo relevance for regeneration are not sufficiently well supported by the data at this stage. Referee#3 agrees in that unequivocal functional in vivo proof is missing and in addition asks you to characterize cell type and molecular features of the cells with regenerative capacity in greater depth.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. Please note however, that we would need strong support from the referees on such a revised version of the manuscript to move towards publication. I agree that it would be essential to consolidate the Lgr5-independent in vivo role of the Atoh+ cells.

REFEREE REPORTS:

Referee #1:

Please find herein my comments for the manuscript (EMBOJ-2018-99984) entitled "Atoh1+

secretory progenitors possess renewal capacity independent of Lgr5+ cells during colonic regeneration" by Ashfaha and colleagues.

In this manuscript the authors use multiple lineage tracing experiments to show that Keratin19 positive but Lgr5 negative colonic cells can regenerate colonic crypts and give again rise to Lgr5+ cells in a DSS induced colitis model. Additional Notch1+- and Atoh1-Cre-ERT mediated lineage tracing experiments show that Notch1 positive absorptive cells do not contribute to epithelial repair, while Atoh1+ secretory progenitors successfully repair the colonic epithelium after injury independent of Lgr5, indicating that Atoh1 secretory cells can acquire important renewal capacity in the context of injury.

The experiments are well performed and nicely documented. The conclusions are justified based on the presented data. They confirm a recent report by Ishibashi et al entitled " Contribution of ATOH1+ cells to the homeostasis, repair and tumorigenesis of the colonic epithelium, (Stem Cell Reports Vol. 10, 27-40, Jan. 2018). Ishibashi et al also uses lineage-tracing experiments (Atoh1-Cre-PGR) to show that Atoh1+ intestinal epithelia cells retain stem cell properties. They compare Atoh1+ cells of the small and the large intestine and also use a DSS-induced colitis model. The experimental set up and the conclusions are very similar. Thus the major problem is a question of novelty, which is clearly compromised.
The authors do cite the Ishibashi paper but only in the introduction (page 3, " Importantly, we and others have also reported that Lgr5+ stem cells are highly sensitive to epithelial injury induced by radiation and colitis Ashafa et al 2015, Ishibashi et al 2018, Yan et al 2102), suggesting that an Lgr5-negaive cell population is responsible, at least in part for colonic regeneration".
Unfortunately, the authors do not discuss similarities, differences and potential novelties of their study in the light of the already published Stem Cell Report paper. In the absence of pointing out the novelties of their study it is difficult to recommend publication in the EMBO journal.

Referee #2:

It has been unclear how the colonic epithelium regenerates after injury such as colitis in the absence of Lgr5+ stem cells. Castillo-Azofeifa et al has identified Atoh1+ cells as the population that regenerate the colon after ablation of Lgr5+ stem cells. The manuscript was simple to follow and the experiments were clear. However, the definitive experiment to support the main argument was not performed, which is explained as follows:

1. The authors mainly relied on the GPF reporter of the Lgr5-EGFP-DTR and Lgr5-GFP-IRES-CreERT2 mouse models to demonstrate the absence and presence of Lgr5+ cells. This is not sensitive enough for detecting Lgr5 expression as GFP is a surrogate marker which requires accumulation to a critical amount to be visible. Hence, Lgr5 expression could be present even before GFP expression is detected. The authors should perform a highly sensitive RNA in situ hybridization method such an RNAscope under two conditions to set the premise for the entire paper:
(i) after the DT treatment regimen in Lgr5-DTR-GFP and
(ii) after DSS treatment to demonstrate unequivocal loss of Lgr5 expression and Lgr5+ cells.
2. Given the title, the definitive experiment to perform would be to induce colitis, initiate tracing and Lgr5+ cell ablation in Atoh1-CreERT2;tdTomato;Lgr5-DTR mice, like the experimental schemes in Fig 1e and 2d. However, the authors had only performed lineage tracing and Lgr5+ cell ablation under homeostatic conditions (Fig 4e-h), and lineage tracing upon injury without ablating the Lgr5+ cells (Fig 4a-d). In vitro organoid passaging was used as a proxy for injury (Fig 5), which cannot replace in vivo DSS treatment.
Hence, after comment #1 has been addressed (ie complete loss of Lgr5 expression verified), please perform this experiment which is necessary to support the title of the manuscript.

Other comments:

3. In Fig 2, the Krt19-CreERT2 mouse was used as the authors "had previously showed that this marker labels intestinal and colonic crypt cells, but excludes rapidly cycling Lgr5+ stem cells". However, in Figure 2 of the Asfaha et al (2015) paper, it was clearly shown that there is a small

number of cells in the crypts that are Lgr5 and Krt19 positive. Please clarify this point.
4. Fig 4g and S1f - It is curious that the tdTomato-traced population appears to shrink between d4 and d8. Could the authors explain this? Does this mean that the Atoh1+ progenitors need to transit through a Lgr5+ state in order to generate progeny for the entire crypt?
5. Overall, the quality of the images is found wanting due to low resolution and the fluorescence tends to be oversaturated. Please replace with images of better quality.
6. The graphs for number of contiguously labeled crypts are confusing because the p-values are indicated for control and DSS-treated conditions for 1 crypt, which is not the question the authors are asking in the manuscript. Since crypt fission and contiguously labeled crypts are used as indicators of progeny expansion, the test of significance should be performed for the bars with >1 contiguously labeled crypts between control and DSS-treated colons.
7. Fig 5c - Unclear how % traced organoids is defined and quantified.

Referee #3:

Summary
The epithelium of the colon turns over every 5 days and this process relies on colonic stem cells. Epithelial turnover in the small intestine has been studied in much depth and the various intestinal stem cell (ISC) pools and molecular players are quite well understood. Moreover, genetic lineage tracing studies have identified other cell types including enterocytes and secretory cells can contribute to regeneration following injury of the small intestine (Tetteh et al., Buczacki et al., Yan et al.) in some cases even when the Lgr5+ ISC pool is ablated. Far less is known about this phenomenon in the colon except a paper published by the co-authors of this manuscript showing that Krt19+ Lgr5- labeled cells contribute to regeneration following radiation-induced injury. In this manuscript the authors use DSS-induced injury model to show that Atoh1-labeled secretory cells contribute to regenerating epithelium and that this process occurs even after ablation of Lgr5 ISCs. This is an important finding and likely to generate interest in the GI community. That being said, there are a number of concerns with the data and interpretations of the data that should be addressed. Do the authors have any evidence as to which secretory cells might be involved in regeneration? Do they have functional evidence in vivo that secretory cells are required for Lgr5-independent regeneration?

Comments
The authors state that their previous findings showed that Krt19 cells are Lgr5-negative. However in the past few years it is clear that there are Lgr5 high and low populations and that they are likely referring to the Lgr5 high cells. What about Lgr5 low cells? Are they Krt19 negative? In general it would be helpful to know if Krt19 is expressed in all non-crypt cells or a subset? For example does Krt19 label all secretory cells?

Krt19 cells contribute to regenerating colonic epithelium and animals in which Krt19 cells have been ablated are less able to repair colonic epithelium following DSS-induced injury. The subsequent lineage tracing data would suggest that secretory cells, not colonocytes, are contributing to the Lgr5-independent repair of the colon in vivo but this was never conclusively demonstrated. One would predict that Atoh1CreERT2; Rosa26tdTomato/DTR would not repair properly, and this would then fully support the authors claim that Atoh1-expressing cells "are critical to colonic epithelial regeneration in the setting of injury".

Do the authors have an idea as to which secretory cell type is involved in Lgr5-independent regeneration following DSS treatment? Are they equivalent to the ones described by Buczacki et al or Yan et al? Do they express Bmi1, Prox1, Neurog3?

Figure 5 is confusing. The title of the figure is misleading as it states that Atoh1 cells are essential for regeneration post- colitis. However, all of the data appear to be in vitro studies, not regeneration in vivo post colitis. In addition the authors state that passaging as an injury model and that it causes expansion of Atoh1 labeled cells. Clearly in vitro culture is stressful, but one cannot conclude that this is an "injury" model. Irradiation in vitro might count as an injury but the colonoids in figure 5 were derived from irradiated mice, not colonoids irradiated in vitro.

1st Revision - authors' response 26th Oct 2018

We are pleased to submit a revised version of our manuscript, entitled "Atoh1+ secretory progenitors possess renewal capacity independent of Lgr5+ cells during colonic regeneration", ID number EMBOJ-2018-99984. We appreciate all three referees' comments, which were helpful in refining the paper, and we are grateful for your continued interest in our work and for all of your help during this process.

We were grateful to read that the referees found our study to be of interest and relevance. We found their comments to be fair and constructive, and by taking them into account during the revision process, we were able to further strengthen the paper. We have addressed the critiques to the best of our ability and have performed all of the experiments the referees proposed; in all cases, we obtained results consistent with our original data. We have also elaborated on the importance and novelty of our findings in the introduction and discussion in response to the advice you provided during our conference call and subsequence emails. Below, we provide a point-by-point response to the reviews and changes that we have made to address the critiques. Finally, we encourage the referees to use the original figure files (ppt format) now provided.

Referee #1:

Please find herein my comments for the manuscript (EMBOJ-2018-99984) entitled "Atoh1+ secretory progenitors possess renewal capacity independent of Lgr5+ cells during colonic regeneration" by Asfaha and colleagues.

In this manuscript the authors use multiple lineage tracing experiments to show that Keratin19 positive but Lgr5 negative colonic cells can regenerate colonic crypts and give again rise to Lgr5+ cells in a DSS induced colitis model. Additional Notch1+- and Atoh1-Cre-ERT mediated lineage tracing experiments show that Notch1 positive absorptive cells do not contribute to epithelial repair, while Atoh1+ secretory progenitors successfully repair the colonic epithelium after injury independent of Lgr5, indicating that Atoh1 secretory cells can acquire important renewal capacity in the context of injury.

The experiments are well performed and nicely documented. The conclusions are justified based on the presented data. They confirm a recent report by Ishibashi et al entitled " Contribution of ATOH1+ cells to the homeostasis, repair and tumorigenesis of the colonic epithelium, (Stem Cell Reports Vol. 10, 27-40, Jan. 2018). Ishibashi et al also uses lineage-tracing experiments (Atoh1-Cre-PGR) to show that Atoh1+ intestinal epithelia cells retain stem cell properties. They compare Atoh1+ cells of the small and the large intestine and also use a DSS-induced colitis model. The experimental set up and the conclusions are very similar. Thus the major problem is a question of novelty, which is clearly compromised.

The authors do cite the Ishibashi paper but only in the introduction (page 3, " Importantly, we and others have also reported that Lgr5+ stem cells are highly sensitive to epithelial injury induced by radiation and colitis Ashafa et al 2015, Ishibashi et al 2018, Yan et al 2102), suggesting that an Lgr5-negaive cell population is responsible, at least in part for colonic regeneration". Unfortunately, the authors do not discuss similarities, differences and potential novelties of their study in the light of the already published Stem Cell Report paper. In the absence of pointing out the novelties of their study it is difficult to recommend publication in the EMBO journal.

We thank the referee for these comments; indeed, some of our observations confirm and validate recent findings reported by Ishibashi et al. 2018 Stem Cell Reports earlier this year, and by Tomic et al. 2018 Cell Stem Cell in August (after our paper was reviewed), pertaining to *Atoh1*+ progenitor cell contribution to epithelial regeneration post-colitis. We have followed the referee's suggestion to more explicitly discuss in the revised manuscript how our work demonstrates important and novel findings, which we outline here:

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1. Previous reports in the literature have focused on the epithelial response to injury of *Lgr5+* cells in the small intestine. In the current study, we have focused on the response of the colonic epithelium to DSS-induced colonic injury.
2. We show, for the first time, that absorptive progenitors, marked by Notch1, lack regenerative capacity in the setting of colonic injury. The inclusion of this data is important to demonstrate that not all colonic epithelial cells are capable of regeneration in the setting of colitis. This set of experiments and results have not been previously reported.
3. As noted by the referee, we also demonstrate that *Atoh1+* secretory cells indeed contribute to colonic regeneration post-colitis. Our data validate the recent findings by Ishibashi et al 2018 Stem Cell Reports, and by Tomic et al. 2018 Cell Stem Cell, that *Atoh1+* cells contribute to colonic regeneration. Despite a similar observation being reported, we would argue it is an important finding that clearly warrants validation by other groups, and in fact, these findings only strengthen our observations presented in this manuscript.
4. In clear distinction to the recent papers by Ishibashi et al. and Tomic et al., who suggest that *Atoh1+* cell overlap with Lgr5-expressing cells, we definitively show that *Atoh1+* secretory cells are distinct from *Lgr5+* cells, yet nevertheless are able to contribute to regeneration in colitis. Thus, our novel findings show that *Atoh1+* cells are distinct from *Lgr5+* cells and contribute to colonic regeneration during colitis and *Lgr5+* cell replacement. The intestinal field will benefit from these new findings, as we move forward in understanding the successive reprogramming events that cells undergo during dedifferentiation and plasticity.
5. In our revised manuscript, we provide new data demonstrating that *Atoh1*-dependent regeneration in colitis takes place without the need for transitioning to an *Lgr5+* state (Figure 4i-k). Our data show that *Lgr5+* cell ablation does not affect the capacity of *Atoh1+* cells to contribute to colonic regeneration in colitis.
6. We demonstrate for the first time that under homeostatic conditions *Atoh1+* single cells have the capacity to form organoids *in vitro*; moreover *Atoh1+* cell plasticity is significantly enhanced by simultaneous ablation of *Lgr5+* cells.
7. Although not a main focus of the present study, we present our analogous findings in the small intestine, where we demonstrate that intestinal *Atoh1+* secretory cells have the capacity to regenerate the epithelium (in response to radiation) independent of *Lgr5+* cells. This is in clear distinction to the previous papers and is the first demonstration to our knowledge that *Atoh1+* cell-dependent intestinal regeneration occurs independently of *Lgr5+* cells.
8. Importantly, in our revised manuscript we demonstrate for the first time that *Atoh1+* secretory cell ablation does not impact the colonic epithelium in homeostasis but in the setting of colitis significantly impairs epithelial regeneration. These new observations presented in Figure 6 clearly demonstrate the requirement of *Atoh1+* cells for proper colonic regeneration *in vivo* and *in vitro*.

Referee #2:

It has been unclear how the colonic epithelium regenerates after injury such as colitis in the absence of Lgr5+ stem cells. Castillo-Azofeifa et al has identified Atoh1+ cells as the population that regenerate the colon after ablation of Lgr5+ stem cells. The manuscript was simple to follow and the experiments were clear. However, the definitive experiment to support the main argument was not performed, which is explained as follows:

1. The authors mainly relied on the GFP reporter of the Lgr5-EGFP-DTR and Lgr5-GFP-IRES-CreERT2 mouse models to demonstrate the absence and presence of Lgr5+ cells. This is not sensitive enough for detecting Lgr5 expression as GFP is a surrogate marker which requires accumulation to a critical amount to be visible. Hence, Lgr5 expression could be present even before GFP expression is detected. The authors should perform a highly sensitive RNA in situ hybridization method such an RNAscope under two conditions to set the premise for the entire paper:
(i) after the DT treatment regimen in Lgr5-DTR-GFP and
(ii) after DSS treatment to demonstrate unequivocal loss of Lgr5 expression and Lgr5+ cells.

We appreciate the referee's suggestion and agree that examining Lgr5 expression post-DSS injury using a completely independent approach is important. To that end, we have used RNAscope single molecule *in situ* hybridization for detection of *Lgr5* expression in colonic tissue. We previously detected a ~15-fold decline in *Lgr5* expression post-DSS, the largest decline in stem/progenitor cell transcript post-DSS, as shown in Figure 1k. Our observations are consistent with the work previously reported by Yan et al. 2012 PNAS, Davidson et al. 2012 Biochimica et Biophysica Acta and Metcalfe et al. 2014 Cell Stem Cell.

We now include in Figure 1c the new RNAscope assay for *Lgr5* mRNA expression. We found that, post-DSS, *Lgr5* expression is completely eliminated in the distal colon. To further validate our observations that *Lgr5*-expressing cells are truly eliminated not only post-colitis, but also following DT ablation, we examined *Lgr5* expression via RNAscope pre and post-DT administration in our *Lgr5-GFP-DTR* mice. As shown in Figure 1l, *Lgr5* expression as detected by RNAscope was completely eliminated following DT ablation of *Lgr5*-expressing cells. Not surprisingly, the effects of DT were consistent across the entire length of the colon, in contrast to DSS, which showed complete loss of *Lgr5* transcript predominantly in the distal colon, its main site of action (Chassaing et al. 2014 Curr Prot Immunol).

2. Given the title, the definitive experiment to perform would be to induce colitis, initiate tracing and Lgr5+ cell ablation in Atoh1-CreERT2;tdTomato;Lgr5-DTR mice, like the experimental schemes in Fig 1e and 2d. However, the authors had only performed lineage tracing and Lgr5+ cell ablation under homeostatic conditions (Fig 4e-h), and lineage tracing upon injury without ablating the Lgr5+ cells (Fig 4a-d). In vitro organoid passaging was used as a proxy for injury (Fig 5), which cannot replace in vivo DSS treatment. Hence, after comment #1 has been addressed (ie complete loss of Lgr5 expression verified), please perform this experiment which is necessary to support the title of the manuscript.

We share the referee's interest in these experiments and we have carried them out as recommended. To address the issue of whether *Atoh1+* progenitor cells can continue to lineage trace post-colitis in the setting of concurrent *Lgr5+* cell ablation using DT, we generated *Atoh1-CreERT2;tdTomato;Lgr5-DTR* mice. As suggested by the referee, we initiated *Atoh1+* lineage tracing and ablated *Lgr5+* cells during colitis. Our new data in Figure 4i-k confirm that *Atoh1+* progenitors continue to show self-renewal capacity and lineage trace in the setting of DT ablation of *Lgr5+* cells.

We agree with the referee's comment that *in vitro* passaging is not equivalent to DSS injury *in vivo*. Thus, as suggested above, we have performed the requested *in vivo* experiment and confirmed that *Atoh1+* cells continue to lineage trace in colitis despite *Lgr5+* cell ablation. Additionally, we have conducted radiation injury experiments in colonic organoids and now report in Figure 5a-c that *Atoh1+* progenitor cells show self-renewal capacity and lineage tracing *in vitro* after radiation-induced ablation of *Lgr5+* stem cells.

Other comments:

3. In Fig 2, the Krt19-CreERT2 mouse was used as the authors "had previously showed that this marker labels intestinal and colonic crypt cells, but excludes rapidly cycling Lgr5+ stem cells". However, in Figure 2 of the Asfaha et al (2015) paper, it was clearly shown that there is a small number of cells in the crypts that are Lgr5 and Krt19 positive. Please clarify this point.

We thank the referee for their observations and agree with their assessment that we previously demonstrated a very rare (<0.01% in the SI and <0.05% in the colon) population of overlapping cells. We also showed that *Krt19* also labels a *Lgr5*-negative cell population that shows regenerative capacity. Importantly, in the previous Asfaha et al. 2015 paper, we showed that *Lgr5+* cells were relatively radio-sensitive when compared to *Krt19+* cells, and this is consistent with our current data shown in Figure 1, where we confirm that *Lgr5+* cells are similarly sensitive to DSS-colitis injury. For this reason, the *Krt19-CreERT2* mouse was initially used to demonstrate that, despite a very small subset of overlapping cells, there is a striking difference in lineage tracing capacity between *Krt19*-expressing and *Lgr5+* cells post-colitis. These data suggest that another cell population within the *Lgr5*-negative compartment of the intestinal crypt allows for regeneration. Our findings in the

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remainder of the paper suggest that this is predominately attributed to the *Atoh1+* secretory progenitor population.

4. Fig 4g and S1f - It is curious that the tdTomato-traced population appears to shrink between d4 and d8. Could the authors explain this? Does this mean that the Atoh1+ progenitors need to transit through a Lgr5+ state in order to generate progeny for the entire crypt?

We share the referee's interest in the observation that the tdTomato+ population appears to shrink at d8 post-DT. We believe that the contraction and localization of the tdTomato-traced population to the bottom of the crypt between d4 and d8 follows a similar phenomenon seen in intestinal regeneration after irradiation, in which there is a proliferative phase (3-4 day post-radiation) followed by a normalization phase and resumption of steady-state (7 day post-radiation) (Kim et al. 2017 Curr Stem Cell Rep). Our data follow the same cell kinetics during the initial regeneration (4 day post-DT), when *Atoh1+* cell expansion is required, and during the subsequent homeostatic restoration (8 day post-DT), when *Atoh1+* cells give rise to new *Lgr5+* stem cells. Furthermore, we have included new data in Figure 4g, showing that 30 days after DT ablation of stem cells, *Lgr5+* stem cells that are newly derived from *Atoh1+* cells are responsible for maintaining homeostasis after the regeneration phase.

5. Overall, the quality of the images is found wanting due to low resolution and the fluorescence tends to be oversaturated. Please replace with images of better quality.

The PDF provided to the referees was compressed in order to reduce the file size for uploading to the journal server. The original image files are of a much higher quality and clarity, and we have now included as large a file as permitted by The EMBO Journal. We have uploaded the original ppt files and will encourage the referees to use these files.

6. The graphs for number of contiguously labeled crypts are confusing because the p-values are indicated for control and DSS-treated conditions for 1 crypt, which is not the question the authors are asking in the manuscript. Since crypt fission and contiguously labeled crypts are used as indicators of progeny expansion, the test of significance should be performed for the bars with >1 contiguously labeled crypts between control and DSS-treated colons.

We agree with the referee's observation and we have clarified our analysis in the text.

7. Fig 5c - Unclear how % traced organoids is defined and quantified.

We thank the referee for this comment. To clarify, we expressed this as the number of organoids that by day 12 (1 week post-radiation) were fully red or completely lineage traced as tdTomato+, relative to the number of organoids in which we could detect Atoh1-tdTomato+ cells. We have also included additional clarification in the methodology.

Referee #3:

Summary
The epithelium of the colon turns over every 5 days and this process relies on colonic stem cells. Epithelial turnover in the small intestine has been studied in much depth and the various intestinal stem cell (ISC) pools and molecular players are quite well understood. Moreover, genetic lineage tracing studies have identified other cell types including enterocytes and secretory cells can contribute to regeneration following injury of the small intestine (Tetteh et al., Buczacki et al., Yan et al.) in some cases even when the Lgr5+ ISC pool is ablated. Far less is known about this phenomenon in the colon except a paper published by the co-authors of this manuscript showing that Krt19+ Lgr5- labeled cells contribute to regeneration following radiation-induced injury. In this manuscript the authors use DSS-induced injury model to show that Atoh1-labeled secretory cells contribute to regenerating epithelium and that this process occurs even after ablation of Lgr5 ISCs. This is an important finding and likely to generate interest in the GI community. That being said, there are a number of concerns with the data and interpretations of the data that should be addressed. Do the authors have any evidence as to which secretory cells might be involved in regeneration?

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We appreciate the referee's insightful review and comments, and their acknowledgment of the novelty of the data in our study, which includes distinction of the role of *Lgr5+* stem cells from *Atoh1+* secretory progenitors in the colon. The referee's question regarding which secretory cell(s) contributes to regeneration is an important one, but also a challenging one to discern, given the limitations of the current transgenic mouse models. In this revision, we demonstrate in Suppl. Figure 2b that *Krt19+* marks a variety of secretory cells, including *Muc2+* goblet cells, *Dclk1+* tuft cells, and *ChgA+* enteroendocrine cells. However, it remains a challenge to attribute either the *Krt19+* or *Atoh1+* lineage tracing capacity observed post-colitis to any one of these different secretory lineages. What we can conclude is that using *Dclk1-CreERT2* mice to lineage trace from tuft cells, we have previously shown that these cells are unlikely to be responsible for the colonic regeneration observed (Westphalen, Asfaha et al, 2014 JCI). Moreover, recent data from Yan et al. 2017 Cell Stem Cell using *Prox1-CreERT2* mice, suggest that enteroendocrine cells, at least in the small intestine, possess regenerative capacity following radiation injury. However, it remains unknown whether this also holds true for cells in the colon.

Do they have functional evidence in vivo that secretory cells are required for Lgr5-independent regeneration?

In the revision, we have included results of a new experiment in which we carried out *Atoh1+* cell lineage tracing studies in the presence of DT administration to ablate *Lgr5+* stem cells during colitis. We refer the referee to our response to Referee #2, question #2 for further details.

Comments
The authors state that their previous findings showed that Krt19 cells are Lgr5-negative. However in the past few years it is clear that there are Lgr5 high and low populations and that they are likely referring to the Lgr5 high cells. What about Lgr5 low cells? Are they Krt19 negative? In general it would be helpful to know if Krt19 is expressed in all non-crypt cells or a subset? For example does Krt19 label all secretory cells?

The referee raises several interesting points. Regarding the overlap between *Krt19* and *Lgr5*-low expressing cells, we previously demonstrated a very rare (<0.01% in the SI and <0.05% in the colon) population of overlapping cells. Please see our response to Referee #2, question #3 for further details.

We also would like to point the referee to our response to Referee #2, question #1, in which we address the issue of how efficient the ablation of *Lgr5*-expressing cells was with DT and DSS using RNAscope. Briefly, in the revised manuscript we have demonstrated that *Lgr5*-expressing cells are completely ablated/eliminated by DT and DSS, suggesting that *Lgr5*-low expressing cells are not likely to significantly contribute to colonic regeneration during colitis.

In regards to *Krt19* expression pattern, we showed in our previous Cell Stem Cell 2015 paper, where we show, using a combination of *in situ*, FACS, a Krt19 reporter mouse and RT-PCR, that *Krt19* mRNA in the intestine is localized to the cells in positions ~4-23. This is similar to the case in the colon where *Krt19* mRNA is localized to the cells above the crypt base, including many of the cells above this level and approximating the top of the crypt. Given this pattern of distribution, *Krt19* does in fact label all secretory cells and we have included new data in Suppl. Figure 2 demonstrating this.

Krt19 cells contribute to regenerating colonic epithelium and animals in which Krt19 cells have been ablated are less able to repair colonic epithelium following DSS-induced injury. The subsequent lineage tracing data would suggest that secretory cells, not colonocytes, are contributing to the Lgr5-independent repair of the colon in vivo but this was never conclusively demonstrated. One would predict that Atoh1CreERT2; Rosa26tdTomato/DTR would not repair properly, and this would then fully support the authors claim that Atoh1-expressing cells "are critical to colonic epithelial regeneration in the setting of injury".

We thank the referee for their suggestions and agree that this experiment would strengthen our point that Atoh1-expressing cells are critical for colonic regeneration. As a result, we generated *Atoh1CreERT2; Rosa26tdTomato/DTR* mice in order to address this point. In Figure 6, we show

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that, upon DT ablation of Atoh1 cells, mice treated with DSS do poorly, with increased weight loss and exacerbation of colitis as determined by histological assessment. Furthermore, using colonic organoids derived from this same mouse line, we also show, for the first time, that DT ablation of *Atoh1+* cells alone does not impact organoid survival, whereas, DT ablation of *Atoh1+* cells in the setting of injury secondary to radiation results in organoid death. These data clearly support our conclusions that *Atoh1*-expressing cells are indeed critical for colonic epithelial regeneration in the setting of injury.

Do the authors have an idea as to which secretory cell type is involved in Lgr5-independent regeneration following DSS treatment? Are they equivalent to the ones described by Buczacki et al or Yan et al? Do they express Bmi1, Prox1, Neurog3?

The referee poses an interesting question, however, we believe this to be beyond the scope of the current study. In the future, we plan to address this question by single cell RNAseq. With our currently available tools, it is difficult conduct genetic studies to know for sure which secretory lineage contributes to regeneration following DSS treatment. (Of note, in the case of *Bmi1*, this has not been reported to mark colonic stem cells nor be expressed in the colon.) Interestingly, although we cannot draw any major conclusions regarding the identity of secretory cell types that contribute to regeneration, our new mRNA expression analysis of secretory markers shows that both *Prox1* and *Neurog3* are most significantly increased upon DSS colitis injury (Suppl. Figure 2c). This would suggest that perhaps secretory cells expressing either or both of these markers could in fact be most important for colonic regeneration.

Figure 5 is confusing. The title of the Figure is misleading as it states that Atoh1 cells are essential for regeneration post-colitis. However, all of the data appear to be in vitro studies, not regeneration in vivo post-colitis. In addition, the authors state that passaging as an injury model and that it causes expansion of Atoh1 labeled cells. Clearly in vitro culture is stressful, but one cannot conclude that this is an "injury" model. Irradiation in vitro might count as an injury but the colonoids in Figure 5 were derived from irradiated mice, not colonoids irradiated in vitro.

We appreciate the referee's comment and have modified Figure 5 to more properly reflect the title. We have now included colonic organoid responses to radiation injury (Figure 5a-c) in addition to the effects of passage (Suppl. Figure 4). Regarding the referee's comment about analysis of colonoids derived from irradiated mice, we apologize for the confusion, as these were in fact images taken from colonoids derived from *Atoh1CreERT2;Rosa26tdTomato* mice, which were then irradiated *in vitro*.
In addition to having addressed the insightful critiques by the referees, which have significantly improved the manuscript, we would like to share with the referees a set of additional experiments that might be valuable to incorporate in the manuscript, if the referees agree. We feel that these additional data consolidate our findings. Briefly:

1. We have knocked out *Atoh1* specifically in the intestine using $Fabpl^{4X\,AT-132}Cre$; $Atoh1^{f/f}$ mice (Shroyer et al. 2007 Gastroenterology), resulting in exacerbation of colonic epithelial damage and impaired recovery post-colitis. This supports our new results obtained from *Atoh1+* cell ablation by diphtheria toxin during DSS treatment, now included in the manuscript.

   Figures for referees have been removed.

2. *Atoh1* knockout resulted in increased cell death, suggesting that *Atoh1* is required for cell survival, which will inevitably affect regeneration.

Figures for referees have been removed.

2nd Editorial Decision 19th Nov 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from referees #1 and #2, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication. Please note that while referee #3 was not able to look back into the work at this time, I have considered his-her points editorially and found them to be adequately addressed as well.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues regarding manuscript formatting as outlined below, which need to be adjusted at re-submission. Please also revisit the integration of recent literature and findings in the discussion as pointed to by referee #2.

REFEREE REPORTS:

Referee #1:

Dear Editors,

I have read the revised manuscript by Klein and colleagues as well as the point by point rebuttal. The authors took my comments into consideration and now discuss how their findings differ from a previous report by Ishibashi et al in the introduction and discussion. In addition, they have also done a great job addressing the comments of the other two reviewers. Taken together, the manuscript improved considerably and is in my opinion now suitable for publication in The EMBO Journal.

Referee #2:

The authors have addressed the experimental concerns and suggestions raised by reviewers. The main issue is that of novelty as two similar papers have been published (as acknowledged by the authors). Nonetheless, the corroboration of the findings with two other recent publications demonstrates convincingly that Atoh1+ progenitors possess the plasticity to regenerate the colonic epithelium upon injury, independent of Lgr5+ stem cells, which would contribute to the intestinal stem cell and regeneration field as a package. One point of note - the authors are well-positioned to summarize and review the findings by the three different groups and they are encouraged to do so in the discussion, which currently does not refer to either of the two publications.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ➜ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In most cases we assumed a minimum of 3 mice would be required to recognize differences between conditions, based upon published data in this system and in other contexts.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In one case one animal was excluded from our analyzes as it was an outlier, which could have been caused by improper treatment or inherent sample physiology. Epithelial damage and lineage tracing between control and treatments were clearly disntinct, therefore, these were used as criteria to determine that this one sample was an outlier.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	To avoid any experimental bias, animals/samples with the appropriate genotype were randomly allocated to treatments. In addition, number of females and males were equally assigned to each condition, as long as availability of mice permitted.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, samples were given a random number and quantifications were performed blind to condition.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We analyzed if our data followed a normal distribution using Shapiro-Wilk normality test.
Is there an estimate of variation within each group of data?	Yes, we used sum-of-squares to determine variation within groups.

Is the variance similar between the groups that are being statistically compared?	For the analyzes we never assumed equal variance between groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Primary antibodies and dilutions used: chicken anti-GFP (1:1000; GFP-1020, Aves Labs), rabbit anti-DCLK1 (1:200; ab31704, Abcam), rabbit anti-chromogranin A (1:100; 20085, ImmunoStar), rabbit anti-Muc2 (1:100; ab76774, Abcam).. Appropriate secondary antibodies from Thermo Fisher Scientific (A11039) were used at 1:1000.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse lines used include combinations of the following alleles or transgenes: Lgr5GFP-IRES-CreERT2 (Jax 008875) (Barker et al, 2007), Lgr5DTR-GFP (Tian et al, 2011), Krt19BAC-CreERT2 (Asfaha et al, 2015), Notch1CreERT2 (Fre et al, 2011), Atoh1CreERT2 (Fujiyama et al, 2009), ROSA26DTR (Jax 007900) (Buch et al, 2005) and ROSA26tdTomato (Jax 007905) (Madisen et al, 2010). Mice were 8-16 weeks of age at the start of each experiment and females and males mice were used.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Rodent work was done in accordance to approved protocols by the Institutional Animal Care and Use Committees at University of California San Francisco and University of Western Ontario.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm the requierements have been meet to the best of our knowledge.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA