EMBO reports

Autophagic state prospectively identifies facultative stem cells in the intestinal epithelium

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1st Editorial Decision **11th Apr 2022**

Dear Dr. Hamilton,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript and the referee reports from The EMBO Journal (attached below). Both referees acknowledge that the findings are of interest. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Thus, we will not require addressing points regarding more mechanistic details experimentally. However, it will be necessary that in a revised manuscript you address all points questioning the main conclusions of the study, and all technical concerns, or points regarding the experimental designs, model systems used, or data presentation.

In this case, you should address the points of referee #1 and the additional comments of referee #2. However, we will not require in vivo data with lineage specific ablation of autophagy related-genes (referee #2), but you should respond to this general concern of referee #2 in the point-by-point response.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

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

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please note our new reference format:
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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the author contributions section.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Kind regards,

Achim

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

This paper shows some interesting results about how functional, non-conventional stem cells can be recruited to serve as stem cells, potentially through the process recently termed paligenosis, involving energetic and structural switches in cell architecture. The authors describe a novel mouse line that allows tamoxifen-induced gene recombination strategies, including genetically monitoring expression from the Chga allele (via dsTomato) and lineage tracing from cells of enteroendocrine differentiation (via a CreERT2-dsTomato allele knocked in to the Chromogranin, Chga, locus) by crossing with LSL-YFP or LSL-LacZ reporter alleles. Using a variety of techniques, they confirm previous results that cells that differentiate along the enteroendocrine lineage (expressing Chga) can serve as functional intestinal stem cells (fISCs). By combining with Tre-H2b-GFP mice that allow pulse-chase following cycling cells to identify "label-retaining cells", the authors show that GFP+ cells (i.e., those that were cycling at time of doxycycline) spawn organoids ex vivo with the same efficiency no matter how long (up to 14d) the chase post-doxycycline. Thus, cells that are "older" (i.e., more days after having proliferated) do not show decreased functional stem cell activity ex vivo, which is in contrast to earlier published data suggesting fISC activity decreases with age. Next, the authors show that cell populations sorted by higher CytoID labeling (correlating with increased intracellular accumulation of autophagic vesicles) form organoids as efficiently as those from constitutively active LGR5+ ISCs and much more efficiently than lower autophagic-vesicle-containing cells. sc-RNA-Seq of CytoID-Hi cells showed enriched Paneth and goblet cells (but not CHGA+ cells). The CytoID-Hi organoid-forming efficiency is increased even within specific cell types (c-Kit+ and Chga+). Finally, they show that LC3-reporter-expressing cells in vivo (ie those with more constitutive autophagosomes) anti-correlate with DNA damage, as measured by gH2AX labeling.

There is a lot that is new and exciting here, namely the potential role for autophagic vesicle accumulation as a way to prospectively predict fISC potential that may be as potent as colony-forming potential even of constitutive, LGR5+ ISCs, new Chga mice that can be used to follow fISCs from endocrine cells, and data suggesting "age" of fISCs is not an important prospective predictor of fISC activity ex vivo.

There are also a few disconnects in the whether the data support the conclusions.

1) The cells with highest autophagic activity seem mostly to be Paneth cells, with some goblet cells in the FACS, and in tissue sections, the LC3+ cells almost all appear to be Paneth cells. Most of the paper is about endocrine cells as fISCs. The authors do show that, among the few CytoID-Hi cells that are endocrine (CHG+), the CytoID-Hi;CHGA+ cells are more potent as fISCs than the CytoID-Lo, but the data leave the strong impression that the CytoID-Hi cells are mostly goblet or Paneth, so the results (or maybe just how the paper is organized) is confusing.

2) Similarly, the fISC aging studies show that LRCs are ~50% CHG+ at the 10d chase, but the functional relationships between endocrine LRCs as fISCs vs other types of LRCs isn't clear

3) Wouldn't it be good to look at age with respect to in vivo injury to see if age has an effect on fISCs when they are more actively being recruited? There may be an issue of timing injury vs ex vivo CFU assays, but this stills seems an important issue.

4) There is some concern in many of the ex vivo CFU/organoid assays that the number of Paneth cells could affect growth. For example, CytoID-Hi populations may be heterogeneous but contain nearly all the Paneth cells, and Paneth cells can support organoid growth, especially if the cell suspension is not entirely pure single-cells, and some cells are doublets with the Paneth cells. But, in any case, how to control for some of the effects in the various fISC ex vivo assays being confounded by Paneth-derived growth signals?

5) The irradiation experiment at the end is important and highly under-analyzed. For one, it seems LC3-RFP is again only in basal cells that look like Paneth cells, whereas gH2AX is in most other cells. Wouldn't it be important to show some LC3-RFP and lack of gH2AX in non-Paneth cells? Also, wouldn't the authors expect the injury to increase autophagy if non-DNA-damaged

cells are recruited by paligenosis to replace damaged cells? This could be examined by a timecourse to follow the LC3-RFP abundance in cells after injury.

6) The cell cycle analyses in Figure 5 are also a bit confusing or disconnected. The cell cycle analyses seem to be forcing all cells into G1, G2M, or S categories, when many (in particular cells that might be recruited via paligenosis) will be in G0. This is clear even in the same figure where 5I shows Ki-67-negative cells, where Ki-67 is a marker of all phases of cell cycle (but not G0).

7) The fraction of fISCs from the aging experiment starts at such low efficiency (~3%), it's hard to know how a decrease might be picked up. Are there positive or negative controls that can be run here to help reassure confidence that the assay has the sensitivity to detect age-dependent differences?

In general, the manuscript is nicely written, but the Introduction should probably reference the Sheahan paper and provide a reference or two on what is known about mTORC1 activity and nutrition in fISCs; also maybe mention the paligenosis concept as all these studies are important for interpreting results. For that matter, paligenosis should be in Abstract or keywords, given its importance in the paper, so the paper can be found on PubMed. There is also a recent review on the topic in intestines that might be cited: PMID: 33969420.

"We asked whether autophagy levels could predict secretory cell plasticity in vitro" -- what are autophagy "levels"? There is autophagy flux, and there are the vesicles and ubiquitin machinery that execute autophagy, but the authors should be careful about generalizing about this complex process where the machinery of macroautophagy (eg autophagosomes) can increase because downstream flux actually decreases (and vice versa).

Minor comments:

"Historically, f-ISCs have been defined using reporter mice with insertion of CreER cassettes into the loci of genes including Bmi1, Hopx, or Lrig1" - I don't think the endogenous Bmi1 locus has been used much in these experiments, as the authors later point out, right?

Referee #2:

Johnson et al propose that intestinal crypt cells with high autophagy activity exhibit plasticity in vitro, proposing autophagy as a cellular state that marks plasticity. The authors use different experimental approaches to verify conclusions that have been made in the intestinal cell plasticity and stem cell field some time ago. For example, they conclude what others have shown regarding the plasticity observed in the secretory cell lineage (Castillo-Azofeifa et al., 2019; Ishibashi et al., 2018; Tomic et al., 2018; van Es et al., 2012). However, the claim that high autophagy activity identifies plastic cells and the conclusion that progenitor cells have high autophagy activity compared to CBCs is not warranted. The data presented here do not support the conclusion that autophagy is an exclusive marker of cells that can revert to a stem cell state; rather the data suggest that autophagy is a marker for identifying TA cells versus CBCs. As useful as in vitro approaches are, the authors used organoids as their only tool to test for cell plasticity. It is important to test their hypothesis in vivo, and lineage specific ablation of autophagy related-genes would provide convincing evidence.

Additional comments:

1. The authors have generated a novel Chga-CreER-2A-tdTomato mouse line. Mice were generated by traditional gene-targeting in ESC, using a Neomycin selection marker. Traditionally, Neo cassette is removed upon generation of founder mice, which was not the case for mutants used in this work. Elimination of Neo cassette is important to prevent potential unwanted effects such as transcriptional dysregulation of unrelated genes, which might compromise subsequent RNAseq-based data obtained using this mouse model. Authors should use appropriate controls to eliminate such an error or address this in the text.

See:
Meyers EN, Lewandoski M and Martin GR (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat Genet 18, 136-141.

Additionally, authors should provide additional evidence on specific labeling of EECs by using additional markers (such as Reg4).

2. Figure S3A,B and 2J - authors examine the plasticity of tdTomato+ cells (faithfully EECs) by organoid formation assay in vitro. Authors report that EECs form organoids with 5x less efficiency, when compared to Lgr5+ derived cells. Their previous results showed an Lgr5+ positive population among tdTomato+ cells (Fig 2. EFG). The authors should provide evidence that tdTomato+ derived organoids did not arise exclusively from Lgr5+ subpopulation.

3. The authors should be consistent in the designation of primer sequences. Four different variants appear in the text (A) 5'F-

GCTCTGAAGGATGCCAGTCA, (B)Primer Chga F: TGTTACCACCACCGCTACTG , (C)forward: 5'-GCTGCTTGACACTGACCCTA and (D) 5'-ATGAGGCGCCATCCTAGTTC-3′

Response to Referee comments

Referee #1:

This paper shows some interesting results about how functional, non-conventional stem cells can be recruited to serve as stem cells, potentially through the process recently termed paligenosis, involving energetic and structural switches in cell architecture. The authors describe a novel mouse line that allows tamoxifen-induced gene recombination strategies, including genetically monitoring expression from the Chga allele (via dsTomato) and lineage tracing from cells of enteroendocrine differentiation (via a CreERT2-dsTomato allele knocked into the Chromogranin, Chga, locus) by crossing with LSL-YFP or LSL-LacZ reporter alleles. Using a variety of techniques, they confirm previous results that cells that differentiate along the enteroendocrine lineage (expressing Chga) can serve as functional intestinal stem cells (fISCs). By combining with Tre-H2b-GFP mice that allow pulse-chase following cycling cells to identify "label-retaining cells", the authors show that GFP+ cells (i.e., those that were cycling at time of doxycycline) spawn organoids ex vivo with the same efficiency no matter how long (up to 14d) the chase post-doxycycline. Thus, cells that are "older" (i.e., more days after having proliferated) do not show decreased functional stem cell activity ex vivo, which contrasts with earlier published data suggesting fISC activity decreases with age. Next, the authors show that cell populations sorted by higher CytoID labeling (correlating with increased intracellular accumulation of autophagic vesicles) form organoids as efficiently as those from constitutively active LGR5+ ISCs and much more efficiently than lower autophagic-vesicle-containing cells. sc-RNA-Seq of CytoID-Hi cells showed enriched Paneth and goblet cells (but not CHGA+ cells). The CytoID-Hi organoid-forming efficiency is increased even within specific cell types (c-Kit+ and Chga+). Finally, they show that LC3-reporter-expressing cells in vivo (ie those with more constitutive autophagosomes) anti-correlate with DNA damage, as measured by gH2AX labeling.

There is a lot that is new and exciting here, namely the potential role for autophagic vesicle accumulation as a way to prospectively predict fISC potential that may be as potent as colony-forming potential even of constitutive, LGR5+ ISCs, new Chga mice that can be used to follow fISCs from endocrine cells, and data suggesting "age" of fISCs is not an important prospective predictor of fISC activity ex vivo.

There are also a few disconnects in the whether the data support the conclusions.

1) The cells with highest autophagic activity seem mostly to be Paneth cells, with some goblet cells in the FACS, and in tissue sections, the LC3+ cells almost all appear to be Paneth cells. Most of the paper is about endocrine cells as fISCs. The authors do show that, among the few CytoID-Hi cells that are endocrine (CHG+), the CytoID-Hi;CHGA+ cells are more potent as fISCs than the CytoID-Lo, but the data leave the strong impression that the CytoID-Hi cells are mostly goblet or Paneth, so the results (or maybe just how the paper is organized) is confusing.

Response

Our initial emphasis on enteroendocrine cells stemmed from multiple published studies discussing facultative stem cell activity from the enteroendocrine population (PMID: 28686870, 28648363). However, we agree with the reviewer that our data suggest CytoID-high cells are mostly goblet and Paneth cells. This is not surprising due to their relative abundance and location in the crypt base compared to Chga+ enteroendocrine cells. Importantly, our main conclusion from this study is that autophagic vesicle content marks fISCs *regardless of lineage*. We therefore modified our text to be inclusive of this point. We opted not to alter the overall narrative, since we demonstrate that CytoID-high Chga+ cells do form organoids at a higher rate than CytoID-low cells, which is inclusive of prior reports that the enteroendocrine lineage can contribute to regeneration. In addition, we provide new data demonstrating that Chga+ cells frequently exist at the crypt base (~24%) and that Chga+ cells with high autophagic vesicle content can be found at the crypt base, albeit at a much lower frequency than Paneth or goblet cells. This is demonstrated through immunofluorescence staining of LC3-RFP+ puncta within crypt base Chga+ cells (Fig. 6F) and included in text in the updated Results section (highlighted in yellow).

2) Similarly, the fISC aging studies show that LRCs are ~50% CHG+ at the 10d chase, but the functional relationships between endocrine LRCs as fISCs vs other types of LRCs isn't clear

Response
The reviewer points to an important question regarding the functional capacity of 10-day chased label retaining cells to behave as facultative intestinal stem cells and to what degree endocrine LRCs exhibit this ability compared to other cell types that comprise this cell population. Previously published work by our group demonstrated that after a 10-day chase, LRCs were composed of roughly 40% Chga+ enteroendocrine cells and 60% cKit+ Paneth cells (PMID: 27237597). Organoid-formation experiments were performed from both cKit- and cKit+ cells within the 10-day chased LRCs in order to determine to what degree cKit- enteroendocrine cells contribute to organoid-formation compared to cKit+ Paneth cells. This study demonstrated that 10-day chased cKit- (EECs) and cKit+ (Paneth) LRCs exhibited comparable organoid-forming efficiencies suggesting that 10-day chased enteroendocrine cells and Paneth cells exhibit similar degrees of plasticity (PMID: 27237597, Figure 6D demonstrated below) [Figures for referees not shown.]

Adapted from Li et al. PMC4961601: Figure 6. *Hopx-CreER* ISCs are functionally distinct from LRCs. (*D*) Ex vivo organoid-forming capacity of single flow-sorted Hopx-Tomato ISCs, ST-LRCs (split into c-Kit^{+} vs c-Kit^{-}), and LT-LRCs, quantified at right. $^{*}P < .01$).

3) Wouldn't it be good to look at age with respect to in vivo injury to see if age has an effect on fISCs when they are more actively being recruited? There may be an issue of timing injury vs ex vivo CFU assays, but this still seems an important issue.

Response
We agree with the reviewer that it is possible that fISC activation via injury may yield new information on the contribution of cell age to regenerative capacity of a given cell, and in fact we considered performing such experiments at the outset of our studies. However, we were unable to determine the proper timing of injury/animal sacrifice following our pulse-chase experiments in a way that results would be interpretable. Published literature has demonstrated that proliferative events following 12Gy irradiation can be observed between 24-72 hours, suggesting that the ideal timepoint for performing organoid-formation experiments on actively recruited facultative intestinal stem cells would be during this timeframe. This could potentially result in misleading conclusions given that 10-day chased LRCs following 12Gy irradiation may differ in cellular

composition compared to non-irradiated control cells due to radiation-induced apoptosis in the intestinal epithelium. Furthermore, given that radiation-induced proliferative events would result in dilution of the GFP label, it is likely that 10-day chased LRCs collected post-irradiation may not be an accurate representation of the facultative intestinal stem cell landscape of the epithelium. We recognize that this is an important question to address, and we are currently optimizing this assay for a future publication.

4) There is some concern in many of the ex vivo CFU/organoid assays that the number of Paneth cells could affect growth. For example, CytoID-Hi populations may be heterogeneous but contain nearly all the Paneth cells, and Paneth cells can support organoid growth, especially if the cell suspension is not entirely pure single-cells, and some cells are doublets with the Paneth cells. But, in any case, how to control for some of the effects in the various fISC ex vivo assays being confounded by Paneth-derived growth signals?

Response
The reviewer makes an excellent point. In Figure 6 we demonstrate using our novel *Chga*$^{CreER\text{-}tdTomato}$ enteroendocrine cell mouse model that CytoID high enteroendocrine cells retain greater organoid-formation efficiency compared to their CytoID low counterparts. This supports the premise that even in the absence of Paneth cells, secretory-lineage cells with high autophagic vesicle content exhibit greater cellular plasticity compared to non-autophagic cells. Furthermore, to demonstrate the rigor of our assays, we have included new panels in Appendix Figure S1 with details of our flow cytometry gating for single cells to demonstrate that doublets are excluded in our organoid formation assays.

5) The irradiation experiment at the end is important and highly under-analyzed. For one, it seems LC3-RFP is again only in basal cells that look like Paneth cells, whereas gH2AX is in most other cells. Wouldn't it be important to show some LC3-RFP and lack of gH2AX in non-Paneth cells? Also, wouldn't the authors expect the injury to increase autophagy if non-DNA-damaged cells are recruited by paligenosis to replace damaged cells? This could be examined by a time course to follow the LC3-RFP abundance in cells after injury.

Response
We agree with the reviewer about the importance of demonstrating autophagic puncta and protection from DNA damage in non-Paneth cells. We have added data to the manuscript showing LC3-RFP+ puncta in both goblet and enteroendocrine cells, supporting the scRNAseq profiling of CytoID high cells. Further, we provide examples of crypt-base goblet and EECs that are protected from DNA damage. Unfortunately, we are unable to stain for LC3-RFP and yH2AX in the same cell, as we are limited by the maximum number of fluorescent channels and antibody species available to us (4). However, we show examples of both high autophagic vesicle content and protection from DNA damage in separate cells of both goblet and EEC lineages, indicating that our observations do extend beyond Paneth cells. These data have been incorporated into Figure 6F.

We further agree that if the intestine were following a paligenosis-like model of regeneration, we would expect a post-injury induction of autophagy prior to mTOR activation. Indeed, our previously published work demonstrated that there is baseline CytoID expression in intestinal epithelial cells and that 12Gy irradiation results in an increase in CytoID mean fluorescence intensity (PMID: 31061170). In addition, studies in *C. elegans* demonstrated that the intestine exhibits robust autophagic flux during homeostasis compared to other tissues (PMID: 26142908). As such, we hypothesize that intestinal epithelial cells that already exhibit high autophagy (and low mTORC1-PMC5919411) at the time of injury are those that are best able to survive and act as fISCs.

6) The cell cycle analyses in Figure 5 are also a bit confusing or disconnected. The cell cycle analyses seem to be forcing all cells into G1, G2M, or S categories, when many (in particular cells that might be recruited via paligenosis) will be in G0. This is clear even in the same figure where 5I shows Ki-67-negative cells, where Ki-67 is a marker of all phases of cell cycle (but not G0).

Response

This is a great comment, but something that is exceedingly complicated to address. The notion of G0, or quiescence, has taken many different meanings over time after its initial description founded upon serum starvation experiments in cultured cells in the 1980s-1990s, which describe a cessation of cell division, along with global suppression of transcription and translation. In modern animal studies of stem cell biology, the term is used to alternatively describe quiescence of non-mitotic cells which are transcriptionally and metabolic dormant, however, it is more often used in the intestinal stem cell field to describe any non-cycling cell. The challenge, which is hotly debated in many fields, is to distinguish truly quiescent, G0 cells from those simply arrested in G1. Ki67 cannot distinguish these states, as *only* proliferative cells in the gut are Ki67+, and all non-proliferative cells are Ki67-negative (Ki67 is continually degraded in both G0 and G1-, e.g., see PMC6108547). For example, all of the enterocytes, which comprise about 90% of the tissue in the small intestinal epithelium, are Ki67-, yet are highly transcriptionally and metabolically active, and thus would be considered arrested in G1 rather than G0. There exists a debate as to whether G0 is a bona fide, molecularly distinct cell cycle state, or simply a variation of a G1 arrest, which we cannot readily speak to. However, in somatic stem cell biology, residence in G0 has been historically associated with greater stem cell potential and quantified based on low rates of translation and transcription and lack of mTORC1 activity. Importantly, and to the reviewer's point, there exists no gene expression program that would enable identification of G0 cells in single cell transcriptomic data- any cells in G0 in our UMAP plots would be called as residing in G1.

Because of these complexities, we avoided any detailed discussion regarding the state of quiescence of fISCs in the current manuscript. However, in quiescent hematopoietic stem cells, muscle satellite cells, and intestinal epithelial cells, stem cell potential and the G0 state have been linked to lack of mTORC1 activity, and activation of mTORC1 is sufficient to drive these stem cell populations out of G0 and into G1 (PMC4065227, PMC4484853, PMC2743144, PMC5100293, PMC4015626). These studies utilize either measures of global transcription or translation to distinguish quiescence from G1, and in our prior studies we demonstrate that fISC quiescence (in Hopx-CreER- or Bmi1-CreER-marked fISCs) is associated with low global transcription via Hoechst-Pyronin flow assays (PMC5100293). Indeed, our prior findings coupled with the well-established negative feedback relationship between mTORC1 activity and autophagy provided some of the impetus for the current study. In addition to low levels of global transcription and translation, numerous studies across multiple tissue types suggests that expression of the Cyclin-dependent kinase inhibitor 1C gene (*CDKN1C*), encoding the p57 protein, is a marker of quiescent stem cells, including in skeletal, hematopoietic, intestinal, and neural stem cells- PMID 21885021, 23481253, 17600112, 35314700. Examining CDKN1C/p57 expression in our datasets reveals that expression of this gene is largely restricted to tuft and enteroendocrine cells: two secretory populations with well-established facultative stem cell activity in the published literature.

* P-value for Cdkn1c violin plot = P = 0.36

7) The fraction of fISCs from the aging experiment starts at such low efficiency (~3%), it's hard to know how a decrease might be picked up. Are there positive or negative controls that can be run here to help reassure confidence that the assay has the sensitivity to detect age-dependent differences?

Response
These experiments included Lgr5+ organoid formation as a gold standard for the field. Consistent with published studies, we found that Lgr5+ cells form organoids at a rate of 5-7%. Organoid formation in published studies from other cell types are in the range of 0.1-5% (PMID: 29848020, 25418730, 19329995). We therefore conclude that 3% efficiency allows for a detection range where a decrease would be observed. Furthermore, the experiments were designed with a technically rigorous approach to ensure direct comparability between samples. For example, organoid growth is variable across experiments. Therefore, for each experimental day, we prep and analyze one sample from each timepoint (d0, d3, d7, d14). This enables internal consistency and although we saw some differences in organoid growth between experimental days, the growth from the different timepoints was consistent on each day. We have added more detail on this technical rigor and reproducibility to the methods section (highlighted).

8) In general, the manuscript is nicely written, but the Introduction should probably reference the Sheahan paper and provide a reference or two on what is known about mTORC1 activity and nutrition in fISCs; also maybe mention the paligenosis concept as all these studies are important for interpreting results. For that matter, paligenosis should be in Abstract or keywords, given its importance in the paper, so the paper can be found on PubMed. There is also a recent review on the topic in intestines that might be cited: PMID: 33969420.

Response
We agree with the reviewer's point that including literature regarding the role of mTORC1 activity in facultative intestinal stem cells, as well as the excellent article by Sheahan et al, in our Introduction is not only important for the historical description of facultative intestinal stem cells but also for interpretation of the data. We have now included this literature in our revised Introduction (highlighted). Furthermore, we now include paligenosis in our keywords. Of note- although paligenosis likely represents a candidate process for the findings presented in this manuscript, the process of paligenosis has not yet been demonstrated in the intestinal epithelium. Given this, our goal was to be inclusive of paligenosis when placing our findings in the broader context, while also being open to other possibilities. This may include tissue-specific differences in those in which paligenosis has been described (stomach, pancreas) and the intestine (for example, differences in secretory cell diversity).

9) "We asked whether autophagy levels could predict secretory cell plasticity in vitro" -- what are autophagy "levels"? There is autophagy flux, and there are the vesicles and ubiquitin machinery that execute autophagy, but the authors should be careful about generalizing about this complex process where the machinery of macroautophagy (eg autophagosomes) can increase because downstream flux actually decreases (and vice versa).

Response
The reviewer makes an excellent point. We have revised our manuscript to ensure that we are clearly stating that we are evaluating autophagic vesicle content and not autophagic flux (highlighted).

Minor comments:
"Historically, f-ISCs have been defined using reporter mice with insertion of CreER cassettes into the loci of genes including Bmi1, Hopx, or Lrig1" - I don't think the endogenous Bmi1 locus has been used much in these experiments, as the authors later point out, right?

Response
Although not all markers that have been utilized to label f-ISCs have utilized the endogenous loci of these genes, the widely used *Bmi1*^{CreER} mouse model developed by the Capecchi group (PMID: 18536716) does target the endogenous Bmi1 locus. PMID:

Referee #2:
Johnson et al propose that intestinal crypt cells with high autophagy activity exhibit plasticity in vitro, proposing autophagy as a cellular state that marks plasticity. The authors use different experimental approaches to verify conclusions that have been made in the intestinal cell plasticity and stem cell field some time ago. For example, they conclude what others have shown regarding the plasticity observed in the secretory cell lineage (Castillo-Azofeifa et al., 2019; Ishibashi et al., 2018; Tomic et al., 2018; van Es et al., 2012). However, the claim that high autophagy activity identifies plastic cells and the conclusion that progenitor cells have high autophagy activity compared to CBCs is not warranted. The data presented here do not support the conclusion that autophagy is an exclusive marker of cells that can revert to a stem cell state; rather the data suggest that autophagy is a marker for identifying TA cells versus CBCs. As useful as in vitro approaches are, the authors used organoids as their only tool to test for cell plasticity. It is important to test their hypothesis in vivo, and lineage specific ablation of autophagy related-genes would provide convincing evidence.

Response
We thank the reviewer for their helpful comments and would like clarify points that were raised by the reviewer and what we presented in the original manuscript.

The reviewer states “the conclusion that progenitor cells have high autophagy activity compared to CBCs is not warranted”. We did not make that conclusion in our original manuscript. Rather, we concluded based upon our data that cells with high autophagic vesicle content are enriched in non-cycling cells of secretory lineages and exhibit an underrepresentation of stem and progenitor cells. We also showed that cells with high autophagic vesicle content within the enteroendocrine and Paneth cell lineages exhibit greater organoid-formation efficiency compared to their low autophagic vesicle counterparts. This is important because there are currently no markers that identify which cells of the secretory lineage can exhibit plasticity when exposed to stem cell niche factors, and thus contribute to regeneration. Lastly, we demonstrated that cells with high autophagic vesicle content are less sensitive to DNA damage.

The reviewer states “The data presented here do not support the conclusion that autophagy is an exclusive marker of cells that can revert to a stem cell state; rather the data suggest that autophagy is a marker for identifying TA cells versus CBCs”. We did not claim that high autophagic vesicle content is an exclusive marker of cells that can revert to a stem cell state. In Figure 5B-C we present single cell sequencing data performed on both CytoID-low and -high cells that demonstrates that CytoID-high cells are enriched in secretory cells and de-enriched in stem and progenitor cells. Furthermore, in Figure 5I, we present data that cells with high autophagic vesicle content are mutually exclusive from the Ki67+ transit-amplifying progenitor population. Published literature also demonstrated that the CBC and TA cell populations are particularly susceptible to DNA damage-induced cell death (PMID: 25609789) further supporting the notion that cells with high autophagic vesicle content are not transit-amplifying progenitor cells.

We agree with the reviewer that additional *in vivo* experiments to test the requirement of autophagy-related genes for cellular plasticity in a lineage-specific manner would further support the conclusions presented in this manuscript. Although autophagy-related genes have been ablated in intestinal epithelial cells in published studies that conclude that autophagy is essential to intestinal regeneration *in vivo* (PMID: 28768191, 32371487) the specific cell type or lineage requiring autophagy for regeneration remains unknown. We did not pursue this experiment for publication in this manuscript for several reasons. First, there is no CreER model enabling target gene ablation in autophagic cells, largely because autophagy is not regulated transcriptionally, and genes encoding proteins involved in autophagy are often ubiquitously expressed. Second, to convincingly determine that autophagy is required within any given lineage, lineage-tracing would need to be performed in conjunction with deletion of an autophagy-related gene, such as *Atg7*. One major limitation to performing this experiment is that the *Rosa26* locus, where, to the best of our knowledge, all lox-stop-lox reporter alleles required for lineage tracing are inserted, is located on the same chromosome and nearby to *Atg7*. Thus, it is not possible to breed a *Rosa26*-based reporter allele into a mouse with a floxed *Atg7* allele and obtain experimental mice with both the *Rosa*-reporter and two *Atg7*-floxed alleles (we tried for 2 years and never

achieved meiotic recombination). Another limitation of such studies is that ablation of autophagy in any one lineage may not be interpretable as other lineages could compensate during regeneration. This outcome is entirely possible in light of our data demonstrating that autophagic vesicle content can select for organoid formation efficiency in multiple lineages (Paneth and enteroendocrine cells).

Additional comments:

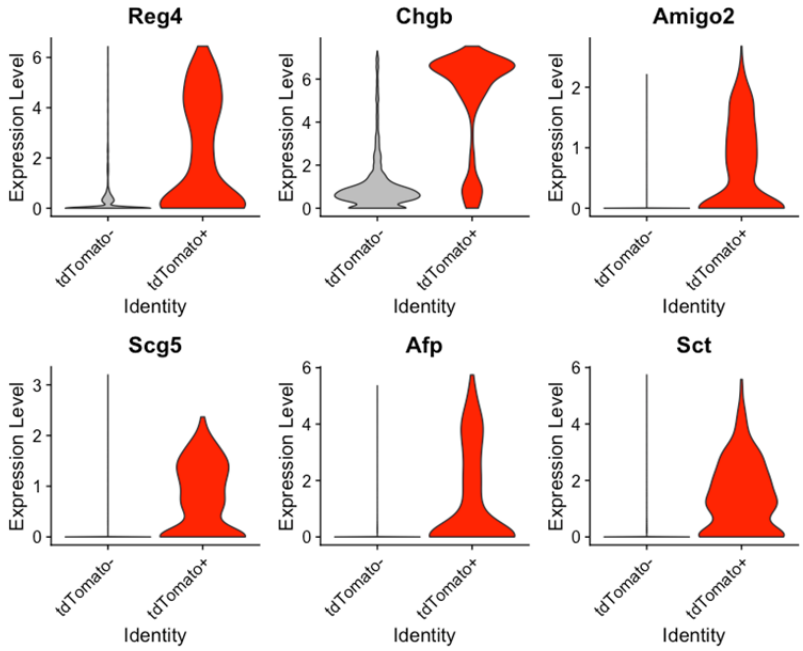
1. The authors have generated a novel Chga-CreER-2A-tdTomato mouse line. Mice were generated by traditional gene-targeting in ESC, using a Neomycin selection marker. Traditionally, Neo cassette is removed upon generation of founder mice, which was not the case for mutants used in this work. Elimination of Neo cassette is important to prevent potential unwanted effects such as transcriptional dysregulation of unrelated genes, which might compromise subsequent RNAseq-based data obtained using this mouse model. Authors should use appropriate controls to eliminate such an error or address this in the text.

See:
Meyers EN, Lewandoski M and Martin GR (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat Genet 18, 136-141.

Additionally, authors should provide additional evidence on specific labeling of EECs by using additional markers (such as Reg4).

Response
We thank the reviewer for pointing out this limitation. While in our study presented here, we did not investigate any transcriptional changes within our Chga+ population, single cell transcriptome profiling did indicate that this population had typical expression of EEC-associated genes. Furthermore, we anticipated that knocking in our CreER-2A-tdTomato cassette, regardless of the neo resistance gene, into the start site of the *Chga* locus would inactivate that copy of the gene, and thus we always kept our mice as heterozygotes. Importantly, *Chga* does not exhibit haploinsufficiency (PMC1159140). We acknowledge that subtle transcriptional changes could be possible with the neo cassette present and added a note of this to the Discussion (highlighted).

To further demonstrate specificity of the *Chga*$^{CreER\text{-}2A\text{-}tdTomato}$ allele, beyond the co-staining of endogenous Chga protein with reporter molecules presented in the original manuscript, we added violin plots showing enrichment of EEC marker genes (Chga, Tph1, Tac1, Neurod1) in the tdTomato+ population to Figure 2I and a list of EEC specific genes enriched in the tdTomato+ population to Figure EV2I. Additionally, we provide violin plots of more EEC marker genes below. We also show that stem related genes (Lgr5 and Ascl2) are enriched in the tdTomato- population (Figure 2I) and that TA related genes are not expressed in the tdTomato+ population (Figure EV2H).



2. Figure S3A,B and 2J - authors examine the plasticity of tdTomato+ cells (faithfully EECs) by organoid formation assay in vitro. Authors report that EECs form organoids with 5x less efficiency, when compared to Lgr5+ derived cells. Their previous results showed an Lgr5+ positive population among tdTomato+ cells (Fig 2. EFG). The authors should provide evidence that tdTomato+ derived organoids did not arise exclusively from Lgr5+ subpopulation.

Response
We agree with the reviewer that it cannot be known which subset of tdTomato+ cells were the ones to form organoids. We quantified that 62/429 tdTomato+ cells expressed some degree of Lgr5 (~15% of cells). Given that tdTomato cells formed organoids at 1/5 the efficiency of Lgr5-High cells, this would necessitate that 100% of the Lgr5 expressing cells present in the tdTomato+ population would need to form organoids in order to fully explain the organoid formation capacity observed with the tdTomato+ population. The probability of this happening is approaching zero given that Lgr5-High expressing cells ordinarily form organoids with an efficiency around 3-5%. Furthermore, this efficiency is calculated among cells expressing high levels of Lgr5 (the brightest 15% of the Lgr5-GFP+ population), and the degree to which this population identified via sorting, and the population of tdTomato+/Lgr5+ cells identified by single cell transcriptomics are similar, is hard to determine. Thus, we acknowledge that it is unknowable which of the tdTomato+ cells were the ones that went on to form organoids and that the results of this assay should be interpreted with this limitation in mind. We have altered the text to highlight this limitation. In the future, we plan to address this experimentally by crossing our Chga-CreER-2A-tdTomato mice to Lgr5-eGFP-DTR mice in order to perform organoid formation assays on a tdTomato+ cell population that is depleted of Lgr5 CBCs.

3. The authors should be consistent in the designation of primer sequences. Four different variants appear in the text (A) 5'F-GCTCTGAAGGATGCCAGTCA, (B)Primer Chga F: TGTTACCACCACCGCTACTG , (C)forward: 5'-GCTGCTTGACACTGACCCTA and (D) 5'-ATGAGGCGCCATCCTAGTTC-3'

Response
We thank the reviewer for pointing out this inconsistency and have modified the text accordingly.

Referee #1, additional cross-comments:

What would strengthen the manuscript is to examine the functional and prognostic role for increased autophagic activity, especially as exhibited within specific subpopulations of cells with stem cell potential and within an in vivo context.

Response

We agree with reviewers that increasing autophagic activity would strengthen the notion that autophagy plays a functional role in subpopulations of cells with stem cell potential and are pursuing such studies in a separate, follow-up manuscript. It should also be noted that studies that indirectly elevate autophagic activity in intestinal epithelial cells through calorie restriction and fasting have demonstrated that these dietary interventions promote injury resistance and regeneration following irradiation (PMID: 29478893, 26686631) further suggesting that the autophagy pathway plays an important role during intestinal regeneration.

Dear Dr. Hamilton,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your work. Referee #1 has some final comments and suggestions to improve the manuscript I ask you to consider for the final revised version of the manuscript.

Moreover, I have these editorial requests I also ask you to address:

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors: https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. It seems, presently not all diagrams have statistical testing.

- Please add scale bars of similar style and thickness to the microscopic images (main, EV and Appendix figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- It seems panel 4B was reused in Fig S1. Please clearly indicate this in the figure legends.

- Please add Appendix Figure S1 to the EV figures. There is room for one more EV figure and then we would not need the Appendix. Please add a legend for this figure to the EV figure legends and remove the Appendix figure legend from the main manuscript file.

- Please remove the referee token from the data availability section and make sure the data are public latest upon publication of the study.

- There seems to be no callout for Fig. EV4 in the manuscript text. Please check and make sure that each figure panel (main and EV figures) is called out separately and sequentially.

- Figure EV1 only has one panel so it does not need the "A" label. Please check.

- Please name the methods section 'Materials & Methods'.

- Please add clear separating white lines to the microscopic images in panel 4D. It seems these are composite images of 4 different images. Please make this clear in the panel by separating the images.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please use this file for further modifications and provide your final manuscript file with track changes, in order that we can see any modifications done.

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Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

I enjoyed the very scholarly discussion with the authors. The response to comments and edits have improved the manuscript, and I have no further issues.

A few additional points:

1) The authors might cite and briefly incorporate this newly published study also showing profound plasticity of stem cell behavior and flow in the crypt: PMID35831497. Among other things, the paper offers more data on LGR5 expression and organoid-forming potential that is in agreement with the authors' work in this manuscript.

2) The authors' point on CDKN1C (p57) and G0 vs G1 is well-taken. I agree with the authors about the complexity and have learned something besides. We who attempt to do cell biology of cells in vivo often find the textbooks (almost exclusively based on cultured, usually malignant, cells) are wrong. What G0 and G1 mean in vivo isn't clear. For what it's worth, it would not be useful to think enterocytes are in G1 simply because they are transcriptionally and metabolically active. Almost all differentiated cells would be so, but the vast majority would either not be able to return to the cell cycle or would take some time doing so. In vivo, there is likely a good deal of organellar rearrangement that is necessary to switch a cell from being physiologically active (eg secreting, absorbing, contracting) to being mitotically active. Of course, the issues are beyond the current manuscript, but they are why it is important to be careful about forcing all cells into G1, S, G2, M categories, as only the latter 3 are reliable in vivo.

3) Interestingly, the authors should be aware that the retooling of architecture to go from large secretory factory to dividing cell that occurs in chief cells in the stomach (i.e., the formally defined paligenosis process) does indeed involve loss of p57 as cells undergo autophagy and before they reignite mTORC1 and pass G1 into S: PMID: 35523142

4) The IF in new Fig. 6F is helpful.

Kudos to the authors for an interesting study. Looking forward to future exciting experiments to follow up these studies!

Referee #2:

The authors have satisfied my previous concerns.

The authors have addressed all minor editorial requests.'

Kathryn Hamilton
Children's Hospital of Philadelphia
Pediatrics
3615 Civic Center Blvd
Abramson 902F
Philadelphia, PA 19146
United States

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The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods (Pages 10-12)

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods (Page 9)

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Material and Methods (Page 11)
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods (Pages 8-9)

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods (Page 12)

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

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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