Regenerative proliferation of differentiated cells by mTORC1-dependent Paligenosis

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Editor: Daniel Klimmeck

Transaction Report:

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

1st Editorial Decision   30th October 2017

Thank you for the submission of your manuscript (EMBOJ-2017-98239) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #3 points out that your claims on a generality of the paligenosis concept and mTORC1-dependent dedifferentiation beyond epithelial tissues are not supported by data currently and asks you to corroborate these claims on other systems. This referee also states the need for you to expand the acinar cell characterization post caerulein treatment. Referee #2 finds that the molecular details of S6 phosphorylation regulation, kinases involved and relative position to KRAS in the signaling are not sufficiently resolved and need more attention. This referee also requests better integration of previous literature linking autophagy to metaplasia and reporting ADM reprogramming, and to expand your discussion on the current findings. In addition, the referees list a number of technical issues on assays used and controls made, that need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I do agree that given the fundamental message of the current study, the work would largely benefit from a deepened discussion of the earlier context and strengthening evidence for a broader relevance of the concept proposed.

RESEREE REPORTS

Referee #1:

This study focuses on cellular reprogramming/plasticity in stomach and pancreas tissue during injury where metaplastic changes occur.
The study shows that mTORC1 activity decreases rapidly following injury to stomach or pancreas and that it later increases and is required for cells to re-enter cell cycle for tissue repair. During the decrease in mTORC1 activity prior to cell cycle re-entry, there is a stage of autophagic cell remodelling that is required to progress to the proliferative phase. The new term "paligenosis" is proposed to denote in general the mechanism of cell dedifferentiation involved in tissue repair, following the above mentioned phases.
The proposed regeneration mechanism is of importance in the domain of regenerative medicine.

Major concerns:

1) In Results section 1, changes in metabolic activity during acute injury are solely examined by immunohistochemical staining of ribosomal protein pS6 in stomach and pancreas, and on Western blot analysis of stomach. In the pictures provided (Fig. 1) it appears that there is a translocation of pS6 immunoreactivity from cytoplasm to the nucleus, especially in the pancreas, which is very odd given that pS6 is a ribosomal protein - this raises questions regarding the specificity of the antibody. It should be specified in Fig. 1-C on how many independent experiments the Western blot measurements are based (n=?), what is represented by the error bars, statistical significance...

2) In the human study it must be specified on how many different human subjects the results are based (n=?).

3) The term "metaplastic gene expression" is used several times in the text but what does it mean (which genes?) in the case of pancreatic acinar cells? This is not shown in this study although the authors want to generalize their findings to stomach and pancreas.

Minor concerns:

4) The cells under study are considered "post-mitotic", however fully differentiated pancreatic acinar cells can be induced to proliferate when exposed to increased levels of e.g. thyroid hormone, or following modulation of TGFß signaling, without need to dedifferentiate. So, the term post-mitotic is not correct for this cell type.

Referee #2:

In this study Mills and colleagues describe a phenomenon they define as Paligenosis. Essentially this describes the process when a secretory cell e.g. an acini needs to go through to become a regenerative cell e.g. ductal cell. In this process a cell needs autophagy/lysozymal degradation to remove the secretory machinery and then needs to upregulate Mtorc1 to re-enter cell cycle and proliferate. The authors use 2 models of metaplasia to investigate this model: Stomach following high dose Tamoxifen and pancreas following Caeruelin. Importantly functional inhibition of mTORC1 using rapamycin or genetic deletion of lysosomal degradation via GNPTAP mutation suppresses this process. Together this makes this a strong study that would be of interest to EMBO readers. I have however a number of points for the authors to consider:

1. P-S6. The authors do not comment on which of the S6 sites are phosphorylated. S6 can be phosphorylated by RSK or S6K with S6K being downstream of MTORC1. The RSK phosphorylation is often downstream of MAPK activity. Given KRAS drives ADM in the pancreas haved the authors looked at this phosphorylation model and whether dedifferentiation is MEK dependent? Do the same factors remain the same downstream of KRAS mutation?. Moreover the group should cite the work of Oded Meyhaus which showed a knock-in of S6 had slowed KRAS induced transformation in the pancreas. Thus not only is mTOR important but phosphorylation of S6

might be more than just a marker.

2. The authors mention a number of studies that show autophagy can affect metaplasia. They dont go into much detail about these studies and should describe them carefully. They should cite the study that in the pancreas following KRAS mutation, loss of ATG7 causes a massive increase in markers of ADM (Rosenfeldt et al Nature).

3. Although these authors are the first to coin the term Paligenosis some studies have considered what might be required for the reprogramming during ADM . For example previous work in pancreas have suggested that RAC1 is required following KRAS to drive ADM to rewire the actin cytoskeleton (gastroenterology). This paper should be discussed.

4. What happens to Ps6 in the GNPTAP knockout following caerulin or HD tamoxifen.

5. Im not completely sure i agree with the discussion of Paligenosis in the intestine. Would a plus 4 cell need to undergo this process to regenerate? I think a paneth cell would but so far the evidence for reprogramming of paneth cells is not so clear. Obviously mTOR has been shown to be critical for intestinal regeneration and this work should be cited but I am not convinced that a similar mechanism need exist in a tissue where many progenitors exist. I would prefer a model where the mTOR element is to needed allow the damaged progenitor cells to enter back into cycle but there is no need for cells to rewire (e.g Lysozymal degradation) themselves in the same manner. I would predict the lysozymal or autophagy mutants would therefore regenerate following irradiation and following DSS.

Referee #3:

The main finding of this study by Willet et al. is that dedifferentiation of highly specialized secretory cells depends on metabolic remodelling via mTORC1 and the cell's autodegradative machinery. The findings are interesting and novel whilst not totally surprising as it is known that during ADM, acinar cells undergo heavy remodelling of their exocrine machinery and loose acinar function. Whilst partially studied in the pancreas, the implication of mTORC1 and GNPTAB is novel for the stomach and helps to understand the molecular mechanism underlying dedifferentiation.

General comments:
1. Whilst interesting, the findings seem oversold by their description under the term "paligenosis". To me, the manuscript presents mechanistic detail for the well-known process of "dedifferentiation". The authors propose "paligenosis" as alternative for the commonly used terms "reversion" and "dedifferentiation". It is unclear in how far introducing yet another term aids scientific communication. "Dedifferentiation" is a well-established, transparent term and commonly used (19,128 PubMed Central entries).
2.The authors advertise the here described dedifferentiation mechanism via mTORC1 as fundamental process. However, all experiments are performed on secretory cells of stomach and pancreas, which the authors admit are highly similar in their biology. The authors give several examples for dedifferentiation of less related cells: glia, lung, heart, etc. without presenting any experimental evidence that a similar mechanism operates in these cells. At least they should discuss if and how their findings could translate to these organs.

Specific comments to experimental design and data presentation:
1) For co-stainings of BrdU with Amy (Fig2D, Fig6G): A co-staining with a nuclear acinar marker (e.g. Mist1) would exclude false-positives introduced by proliferating stromal and terminal ductal cells that are interspersed between acini and are known to proliferate upon pancreatic damage and during regeneration.
2) Suppl. Fig2 and 6. The authors should include stainings and quantifications of apoptosis.
3) Fig3A. The authors should label the fluorescence more clearly in each image. It is also not clear what the arrow denotes in the image 3.
4) The authors propose that the initial inhibition of mTORC1 upon injury triggers autophagy, but the

evidence seems missing. The authors could test this notion by using a mTOR activator just before injury induction and see if it could prevent autophagy and subsequent reprogramming.

5) Fig5. The authors should add time-course (e.g. from day 1 to day 5) quantitative data for lysosome or LC3-GFP for pancreas to show the kinetics of autophagy during ADM.

6) Suppl. Fig6B. The authors should characterise the acinar remnants in GNPTAB-/- mice 2 weeks post caerulein treatment better (Mist1, SOX9, Krt19 and Amy stainings) as the acinar cells look strikingly different compared with the 5d time point. At 2 weeks, acinar cells display an atypical morphology, which raises questions about their functionality. To see if this is relevant to the mice, the authors could add data on exocrine insufficiency (stool analysis) and body weight.

1st Revision - authors' response   22nd December 2017

Point by Point Response to Referees:

Editor: *"In particular, referee #3 points out that your claims on a generality of the paligenosis concept and mTORC1-dependent dedifferentiation beyond epithelial tissues are not supported by data currently and asks you to corroborate these claims on other systems. This referee also states the need for you to expand the acinar cell characterization post caerulein treatment. Referee #2 finds that the molecular details of S6 phosphorylation regulation, kinases involved and relative position to KRAS in the signaling are not sufficiently resolved and need more attention. This referee also requests better integration of previous literature linking autophagy to metaplasia and reporting ADM reprogramming, and to expand your discussion on the current findings. In addition, the referees list a number of technical issues on assays used and controls made, that need to be addressed to achieve the level of robustness needed for The EMBO Journal.*

I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I do agree that given the fundamental message of the current study, the work would largely benefit from a deepened discussion of the earlier context and strengthening evidence for a broader relevance of the concept proposed."

     ***Response**: We are glad that the editorial team finds the referee comments reasonable and that we have been invited to revise. As mentioned above, we believe we have answered all the principal concerns highlighted editorially in our revised manuscript. As those concerns derived from the referee comments, we will address them below in our response to the referees.

Referee 1: *"This study focuses on cellular reprogramming/plasticity in stomach and pancreas tissue during injury where metaplastic changes occur. The study shows that mTORC1 activity decreases rapidly following injury to stomach or pancreas and that it later increases and is required for cells to re-enter cell cycle for tissue repair. During the decrease in mTORC1 activity prior to cell cycle re-entry, there is a stage of autophagic cell remodelling that is required to progress to the proliferative phase. The new term "paligenosis" is proposed to denote in general the mechanism of cell dedifferentiation involved in tissue repair, following the above mentioned phases. The proposed regeneration mechanism is of importance in the domain of regenerative medicine."*

     ***Response**: We are happy that the Referee agrees with us about the importance of the study and hope to answer any remaining concerns in our revised manuscript.

Major concerns:

1) "In Results section 1, changes in metabolic activity during acute injury are solely examined by immunohistochemical staining of ribosomal protein pS6 in stomach and pancreas, and on Western blot analysis of stomach. In the pictures provided (Fig. 1) it appears that there is a translocation of pS6 immunoreactivity from cytoplasm to the nucleus, especially in the pancreas, which is very odd given that pS6 is a ribosomal protein - this raises questions regarding the specificity of the antibody."

***Response**: We agree with the referee that it would be odd to have pS6 in the nucleus. Accordingly, we don't actually see pS6 within the nuclei of viable cells. It is, however, associated with the rough ER that wraps tightly around the nucleus. In cells with high nuclear to cytoplasmic ratios and scant cytoplasm, because these perinuclear rings of rER harbor the majority of pS6+ ribosomes, this pattern can look nuclear. However, it is actually perinuclear. The staining pattern is obvious when looking at the nuclear channel and pS6 channel independently under the microscope (or in optical sections). There is also some cell death by d5, in pancreas, which we address below in other comments. Occasional condensed nuclear fragments do label with pS6, but no viable cells show this pattern. To conserve space in the manuscript we have not included individual channels for Fig1A and C to parse this. We show below split channels from a zoomed in portion of the pancreas image used in the manuscript. Note that all the strongly pS6+ cells have cytoplasmic staining, (i.e. there is a hole in pS6 label where the nucleus is). There are some fragments of nuclei that stain (e.g. arrow); these seem mostly to be in the zone of the infiltrating cells within the stroma, not the epithelial cells that concern us currently. The stomach is similar, though there is far less death of pS6+ cells, and so these nuclear fragments are not seen as often, whereas there are many cells with scant cytoplasm that are discussed above. We also refer the referee to comments below about our additional studies of the pS6 antibody.

"It should be specified in Fig. 1-C on how many independent experiments the Western blot measurements are based (n=?), what is represented by the error bars, statistical significance... "

***Response:** In the revised manuscript, we now have n of 3 separate experiments (5 separate mice, 1 blot/mouse) and we complete an appropriate statistical analysis for the 240/244 antibody, the original antibody we used in the manuscript. We have also included a quantitative analysis from Western blots for an additional pS6 (235/236) antibody to give a more comprehensive analysis of the changes in phosphorylation on pS6 in our stomach injury model. The 235/6 changes in
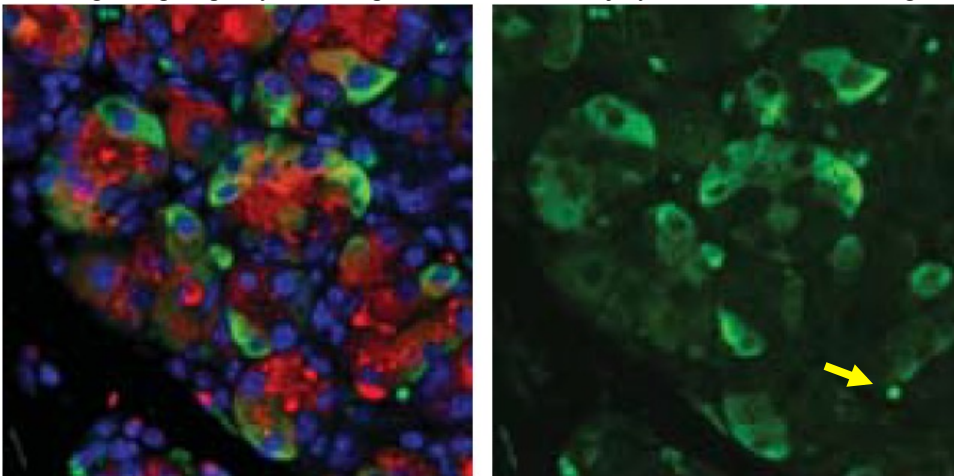
phosphorylation are consistent with the behavior of the 240/244 antibody. See below for additional discussion.

***Revision in Manuscript:** We have updated figure 1C to include additional n with the 240/244 pS6 antibody (5 mice, 3 experiments) and an addition pS6 antibody (235/236 – 2 mice, 2 experiments). Statistical analysis with both antibodies is an ANOVA with post-hoc Dunnett. We have updated the materials and methods and figure legend to reflect these changes.

2) *"In the human study it must be specified on how many different human subjects the results are based (n=?)."*

***Response and Revision**: We have cleared up the context of Fig 3. Fig 3A is a representative image from an analysis of 34 (Lennerz et al., *Am J Pathol*) and 10 (Radyk et al., *Gastroenterology*) separate, curated gastric clinical samples. We now provide this detailed information in the text where we first discuss this analysis in the Results and in the Materials and Methods. Fig 3B human

subject numbers are listed in the figure with detailed information for the patient demographics in Appendix table S2.

3) "The term "metaplastic gene expression" is used several times in the text but what does it mean (which genes?) in the case of pancreatic acinar cells? This is not shown in this study although the authors want to generalize their findings to stomach and pancreas."

***Response and Revision:** We meant "metaplastic gene expression" to mean the activation of the genes that allow cells to be identified as metaplastic (i.e. no longer normal). Generally, such change in gene expression is indicated by using selective markers of metaplasia: in the stomach, for example, one identifies metaplasia by the co-expression of GSII epitope and GIF, or de novo expression of CD44v and SOX9 in cells at the base. In pancreas, we have used metaplastic markers such as CK19/Amylase overlap and PDX1 expression, for example. In any case, we have removed or clarified our usage of this term throughout, because detailed analysis of metaplastic gene expression is not the focus of the manuscript.

Minor concerns:

4) "The cells under study are considered "post-mitotic", however fully differentiated pancreatic acinar cells can be induced to proliferate when exposed to increased levels of e.g. thyroid hormone, or following modulation of TGFß signaling, without need to dedifferentiate. So, the term post-mitotic is not correct for this cell type."

***Response and Revision:** Agreed. These cells can self-duplicate under certain conditions and, rarely, in homeostasis. Indeed, in other experiments in the lab, we have observed that BrdU-labeling over weeks does reveal some duplicating chief and acinar cells even without injury. But, as the reviewer notes, that self-duplication process is not what we are studying. In the interest of language precision, we have removed the term post-mitotic wherever it was inappropriate.

Referee 2:

"In this study Mills and colleagues describe a phenomenon they define as Paligenosis. Essentially this describes the process when a secretory cell e.g. an acini needs to go through to become a regenerative cell e.g. ductal cell. In this process a cell needs autophagy/lysozymal degradation to remove the secretory machinery and then needs to upregulate Mtorc1 to re-enter cell cycle and proliferate. The authors use 2 models of metaplasia to investigate this model: Stomach following high dose Tamoxifen and pancreas following Caeruelin. Importantly functional inhibition of mTORC1 using rapamycin or genetic deletion of lysosomal degradation via GNPTAP mutation suppresses this process. Together this makes this a strong study that would be of interest to EMBO readers. I have however a number of points for the authors to consider: "

***Response:** We are happy that the Referee thinks this is a strong study and of interest to the *EMBO Journal*. We hope to answer any points for consideration.

1A. "P-S6. The authors do not comment on which of the S6 sites are phosphorylated. S6 can be phosphorylated by RSK or S6K with S6K being downstream of MTORC1."

***Response**: Excellent point. We originally used the 240/244 pS6 antibody, which to our knowledge is only modified by pS6K – while RSK, downstream of MAPK activity, has been shown to modify 235/6 (Roux et al., 2007). However, we agree that the original manuscript could be improved by both describing the experiments more explicitly and increasing the robustness of our analysis. Per Referee comments, we have now included Western and IF analysis of the 235/6 pS6 antibody as well. We find the behavior of the 235/6 phosphorylation to be equivalent to the pS6 240/244 site in response to injury and in the fact that it is also fully abrogated by rapamycin, indicating that S6 phosphorylation in our metaplastic injury models is largely due to pS6K activity, though we cannot rule out, of course, an indirect mechanism wherein mTORC1 may indirectly affect RSK.

In any case, we do want to emphasize that the purpose of our study was to interrogate the behavior of mTORC1; thus, what is key is that S6 phosphorylation be an accurate surrogate for mTORC1 activity. Accordingly, rapamycin eliminates both the homeostatic and metaplastic pS6, using either antibody, indicating we have effectively inhibited mTORC1 activity. In our hands, the 240/244 antibody gives stronger signal; it also is thought to be specific for the most direct mTORC1 effect, via S6 Kinase. Thus, we continue to use the antibody recognizing 240/244 for the experiments in the study, outside the ones dealing specifically with the epitope issue. On the other hand, we are also eager to begin to understand the various signaling pathways that are active during paligenosis in general and in addition to the different inputs into pS6. Toward that end, we note that we have previously published that ERK signaling is elevated following tamoxifen injury, which could feed into S6 phosphorylation on 235/236 via RSK. However, the timing and location of ERK activation does not coincide with pS6 changes (Khurana et al., 2013).

***<u>**Revision in Manuscript:**</u> We have added a 235/236 Western Blot data in Figure 1C and performed immunofluorescence analysis of the behavior of 235/236 pS6 in response to Rapamycin/injury which we demonstrate in an augmented Fig EV1. We have also included additional information and citations on the regulation of pS6 in the Results.

1B. *"The RSK phosphorylation is often downstream of MAPK activity. Given KRAS drives ADM in the pancreas have the authors looked at this phosphorylation model and whether dedifferentiation is MEK dependent?"*

***<u>**Response**</u>**: As mentioned above, we have published on ERK activity early after injury (6 to 24-hour range) in the isthmus zone (i.e., not the region where chief cells undergo paligenosis) following tamoxifen injury. Blocking ERK activity in this early window within the isthmus decreases proliferation (Khurana et al., 2013). We have not fully investigated whether MAPK/ERK signaling is required for metaplasia formation in the tamoxifen model at later time points. In the pancreas, MEK inhibition in cerulein-induced injury does not appear to block ADM formation (Collins et al, 2014), but does block further cancer progression in the context of K-Ras mutations. See our description of the 235/6 phosphorylation event above to investigate input from MAPK/ERK/RSK pathway. We plan future experiments on the relationship between ERK signaling, stem cells, paligenosis, and mTORC1. In fact, these experiments are the subject of a grant proposal just submitted by the senior author in an effort to fund exactly such a future analysis.

1C. *"Do the same factors remain the same downstream of KRAS mutation?"*

*****Response:**** Publications in the pancreas and intestine have demonstrated that both autophagy (Rosenfeld et al, 2013) and mTORC1 activity are required for oncogenic behavior downstream of *KRAS* and *APC* mutations (Khalaileh et al., 2013; Morran et al., 2014; Faller et al., 2015).

*****Revision in Manuscript**: We have provided a more clear understanding of this literature in the Discussion section of our manuscript.

1D. *"Moreover the group should cite the work of Oded Meyhaus which showed a knock-in of S6 had slowed KRAS induced transformation in the pancreas. Thus not only is mTOR important but phosphorylation of S6 might be more than just a marker."*

*****Response and Revision:**** Dr. Meyhaus's study (Khalaileh et al., 2013) mechanistically links the phosphorylation of S6 with the progression of carcinogenesis. We agree with the referee that this study alongside other studies describing the role of mTORC1 activity downstream of oncogenesis (mentioned above) are an important aspect of the literature that complements and supports our own current findings of mTORC1 activity in potentially precancerous lesions. Thus, we have happily added these papers to our Discussion.

2. *"The authors mention a number of studies that show autophagy can affect metaplasia. They don't go into much detail about these studies and should describe them carefully. They should cite the study that in the pancreas following KRAS mutation, loss of ATG7 causes a massive increase in markers of ADM (Rosenfeldt et al Nature)."*

*****Response and Revision**: Agreed. We now provide a more robust analysis of previous papers indicating a role for autophagy and mTORC1 in the context of regeneration/metaplasia and downstream of oncogenic mutations in our revised Discussion.

3. *"Although these authors are the first to coin the term Paligenosis some studies have considered what might be required for the reprogramming during ADM. For example previous work in pancreas have suggested that RAC1 is required following KRAS to drive ADM to rewire the actin cytoskeleton (gastroenterology). This paper should be discussed."*

*****Response and Revision:** We have added this paper to our Discussion.

4. *"What happens to Ps6 in the GNPTAP knockout following caerulin or HD tamoxifen."*

*****Response**: We had not thought to do these experiments, which, in retrospect, make a lot of sense. We thank the Referee for suggesting them. We have now addressed this question in the stomach, which is the more synchronous model. *Gnptab*^{−/−} have normal pS6 during homeostasis and also shut off pS6 following HD TAM damage like wildtype mice. However, *Gnptab*^{−/−} mice did not reactivate pS6 at peak metaplasia stages. Thus, both the re-expression of progenitor ("metaplastic") gene phase and the mTORC1-dependent cell cycle re-entry phase do not occur, which is the expected result based on our model.

*****Revision in Manuscript:** We have provided analysis of the behavior of pS6 in *Gnptab*^{−/−} at peak metaplasia stages following HD TAM, and show that pS6 fails to reactivate in chief cells following injury in a new Supplemental figure (Fig. EV7).

5. *"Im not completely sure i agree with the discussion of Paligenosis in the intestine. Would a plus 4 cell need to undergo this process to regenerate? I think a paneth cell would but so far the evidence for reprogramming of paneth cells is not so clear. Obviously mTOR has been shown to be critical for intestinal regeneration and this work should be cited but I am not convinced that a similar mechanism need exist in a tissue where many progenitors exist. I would prefer a model where the mTOR element is to needed allow the damaged progenitor cells to enter back into cycle but there is no need for cells to rewire (e.g Lysozymal degradation) themselves in the same manner. I would predict the lysozymal or autophagy mutants would therefore regenerate following irradiation and following DSS."*

*****Response and Revision**: We thank the Referee for the insightful comments. These are obviously early days in considering what exactly the conserved program of cell cycle re-entry will turn out to be. Perhaps +4 cells are a different type of progenitor that potentially doesn't need to go through the first stage of paligenosis, because there would be little architecture to "rewire". Perhaps another key issue is whether cells need to recycle existing structure to generate amino acids and other macromolecules to subsequently re-activate mTORC1; +4 cells might acquire such building blocks via surface transporters or different interactions with underlying capillaries that might supply them. Perhaps the cells already have genes like SOX9 expressed and, thus, do not need to go through the autodegradation phase if that is necessary for subsequent gene induction. Conversely, one could speculate that Paneth cells, if they are recruited for cell cycle re-entry, might go through the full, 3-stage model we describe in the current manuscript. We hope the Referee will agree that the current paper can serve at the very least as a potential roadmap for all such future studies. In any case, per Referee suggestion, we have amended the Discussion of the manuscript to posit the additional idea that certain cells, like the +4s in the intestine, may not need to go through an autodegradative phase because they may be constitutively more poised to respond to injury.

Referee 3:

"The main finding of this study by Willet et al. is that dedifferentiation of highly specialized secretory cells depends on metabolic remodelling via mTORC1 and the cell's autodegradative machinery. The findings are interesting and novel whilst not totally surprising as it is known that during ADM, acinar cells undergo heavy remodelling of their exocrine machinery and loose acinar

function. Whilst partially studied in the pancreas, the implication of mTORC1 and GNPTAB is novel for the stomach and helps to understand the molecular mechanism underlying dedifferentiation."

***Response:** We thank the referee for the favorable assessment of the impact of our work and pointing out the novelty of our findings for the stomach.

General comments:

1. "Whilst interesting, the findings seem oversold by their description under the term "paligenosis". To me, the manuscript presents mechanistic detail for the well-known process of "dedifferentiation". The authors propose "paligenosis" as alternative for the commonly used terms "reversion" and "dedifferentiation". It is unclear in how far introducing yet another term aids scientific communication. "Dedifferentiation" is a well-established, transparent term and commonly used (19,128 PubMed Central entries)."

***Response and Revision:** Three years ago, we might have agreed with the Referee that the word "dedifferentiation" was a sufficient container term within which we could discuss the cellular mechanisms that concern us here. However, reviewers of our earlier papers pointed out that "dedifferentiation" has limitations if we wanted to use it the way we had been using it and the way the Referee now suggests. For one, many disagree about whether the process of metaplasia we study in the stomach is actually dedifferentiation. Many think it is a transdifferentiation. To prove it is a dedifferentiation, we would have to show that the cells have become either akin to some specific fetal cell type and/or regain multipotent progenitor status. Another issue with "dedifferentiation" is that it may mean one thing to developmental biologists, but it means something else to pathologists and oncologists. "Dedifferentiation" in tumors just means a change to a more aggressive phenotype with marked cytological atypia and bizarre patterns of tissue markers but does not imply what we (or the Referee we assume?) mean in the current study.

To examine this issue with some actual data, we looked at ~80 manuscripts in PubMed, ~half being the most recent and ~half being the earliest uses of the term, to get some idea about how it has actually been used in the literature. Of the most recent, only 54% used it in the sense of a mature cell regaining a more precursor/progenitor status. The rest were about dedifferentiation in tumors or in rarer other contexts or in some combination. Of the earliest, only ~40% used it in the sense we mean in the current manuscript. Thus, though there is some trend towards using dedifferentiation the way we would want to use it, the term clearly is not particularly specific.

In any case, we would argue that what we are trying to do here is to nudge the field into considering the mechanisms whereby cells change cell fate and introduce the idea that these mechanisms, despite varying starting and ending states might be conserved. In other words, whether a "reprogramming" or a "dedifferentiation" or "transdifferentiation" or "reversion", the basic stages may be the same across tissues and species. The existing terms like dedifferentiation and transdifferentiation do not focus on the process but on where the cells wind up. Perhaps there is some parallel here to why the term "apoptosis" was coined. It is not simply that cells died; it was that they used, in some cases, a conserved program to do so.

In the Revised Introduction and Discussion, we attempt to argue the case for a new term for the cellular program that concerns us here and thank the Referee for pointing out that we had not adequately done so in the original manuscript.

2. "The authors advertise the here described dedifferentiation mechanism via mTORC1 as fundamental process. However, all experiments are performed on secretory cells of stomach and pancreas, which the authors admit are highly similar in their biology. The authors give several examples for dedifferentiation of less related cells: glia, lung, heart, etc. without presenting any experimental evidence that a similar mechanism operates in these cells. At least they should discuss if and how their findings could translate to these organs."

***Response**: We agree with the Referee that acinar and chief cell biology share many features and we share the excitement about the idea that mTORC1 controls a fundamental aspect of dedifferentiation in diverse tissues and organisms. We hope the Referee and editors will understand that considering the multiple steps of paligenosis in both pancreas and stomach in parallel has

resulted in a substantial amount of data and a large manuscript as it is. However, given the importance of the issue, for the revised manuscript we have both increased the number of injury models we examine and expanded and refined our analysis of our work with respect to previously published work.

Given time and space constraints, we sought two models that were readily available to us to further examine in re: mTORC1 and cell cycle re-entry during dedifferentiation/regeneration. One of our original co-authors had been working with tunicamycin-injured kidneys, which had previously been shown to involve recruitment of tubular cells into the cell cycle during repair. We examined the tissue blocks we had available from these experiments to determine if S6 phosphorylation occurred in the cell population recruited into the cell cycle. Furthermore, collaborators of ours in the Gastroenterology Division, Drs. Davidson and Blanc, had tissue sections available from partial hepatectomies at the time point of maximal recruitment of mature hepatocytes into the cell cycle, so we also examined pS6 in those sections.

Previous work had shown decades ago that partial hepatectomy induces – just as we observe in stomach and pancreas – massive lysosomal upregulation following injury (around 4 hours post-injury; Becker and Lane, 1965). mTORC1 activity has been shown to be upregulated prior to (12 hours) and during peak proliferative stages following partial hepatectomy. Furthermore, treatment with rapamycin blocks cell cycle progression following partial hepatectomy; this effect was shown to be dependent on pS6K activity (et al., 2011).

Tunicamycin induces acute kidney injury associated with proximal tubule damage. Proximal tubule cells that survive dedifferentiate and proliferate to repair damage (Chang-Panesso and Humphreys, 2017). mTORC1 has been shown to be activated following tunicamycin induced injury, but it is unclear whether it has effects on injury-induced proliferation in this system, and the specific cell population with increased pS6 had not been assessed (Kato et al., 2012), which we do now for the revised manuscript.

***Revision in Manuscript:** We have now added an additional Figure (Fig EV2) with both liver and kidney data showing that in both systems, it is specifically the cells recruited back into the cell cycle that have elevated mTORC1/pS6. Thus, our new results support a key role for mTORC1 in dedifferentiation in cells that are not classical secretory epithelial cells. In the revised Discussion, we have now also greatly expanded our analysis of our data within the context of the literature in which the mechanisms of cellular reprogramming had been considered in isolation. We now point out that the literature plus our current experiments reveal that the first mature hepatocyte response to hepatectomy is massive autophagy/lysosome activation followed by increased SOX expression, and then proliferation, which is dependent on mTORC1 activation. We further mine the literature on reprogramming of adult cells into iPSCs to support the notion that the early steps depend on autophagy, and late stages depend on mTORC1 and proliferation. Finally, we now discuss how glial dedifferentiation is known to first involve autophagy, though a role for mTORC1 has, to our knowledge, not been assessed. And we discuss our current kidney results and the literature indicating a role for mTORC1 in post-injury proliferation, though, to our knowledge, autophagy has not yet been examined in that system.

We hope the editors and referees will allow that, at some point, we have to cut off what we can do in the current manuscript and leave further examination of all the tissues and species in which paligenosis might occur to future studies that may be stimulated by – and increase the impact of! – the current one.

Specific comments to experimental design and data presentation:

1) "For co-stainings of BrdU with Amy (Fig2D, Fig6G): A co-staining with a nuclear acinar marker (e.g. Mist1) would exclude false-positives introduced by proliferating stromal and terminal ductal cells that are interspersed between acini and are known to proliferate upon pancreatic damage and during regeneration."

***Response**: We agree with the referee that the most rigorous method to count acinar-specific proliferation would be to co-stain with an acinar specific nuclear marker alongside BrdU. However, to our knowledge, acinar-specific nuclear markers with suitable antibodies (*Ptf1a*, *Mist1*, *Gata4*) are

shut off early during the paligenosis process (we and others have shown this in previous publications and also in experiments for the current study). Other markers known to become activated within acinar cells in this time window (*Sox9*, *Pdx1*, *Hnf1b*, *Onecut1*, etc.) are not specific to acinar cells as they also label pre-existing ductal cells. In all experiments, we use amylase as a co-label, which is not expressed by stromal and ductal cells. This is relatively standard practice in the field. The senior author is also a pathologist who has analyzed the data presented. It is relatively straightforward – even in massive injury conditions – to distinguish exocrine epithelial cells from stromal or infiltrating immune cells by histological features alone. Contribution from centro-acinar cells or terminal duct cells that may express amylase cannot be fully ruled out, but many previous lineage tracing studies, including our own using *Mist1-Cre^{ERT2}* have shown that the vast majority of the proliferating, amylase+ epithelial cells derive from reprogramming acinar cells.

2) "Suppl. Fig2 and 6. The authors should include stainings and quantifications of apoptosis."

***Response:** We thank the reviewer for suggesting this line of experiments. We have now done cleaved caspase staining in the stomach and pancreas models.

In the stomach, that has helped us to confirm our suspicion that the reason for dropout of the chief cell zone in many *Gnptab$^{-/-}$* bases is likely due to apoptosis of chief cells that cannot undergo paligenosis. As we have previously reported (Huh et al. *Gastroenterology*, 2012; Radyk et al. *Gastroenterology*), chief cells largely do not die in HD-Tam; only parietal cells die. Our cleaved caspase analysis confirms this in the bases of WT HD TAM Day 3 and Rapamycin treated HD TAM Day 3 tissue. The differences between those latter situations and the *Gnptab$^{-/-}$* mice in HD-Tam are qualitative, as whole bases can frequently be seen filled with apoptotic chief cells in the *Gnptab$^{-/-}$* mice, so we hope the Referee will allow that quantification of this more or less control experiment achieving the expected result would be superfluous.

Apoptosis of acinar cells is a characteristic of acinar cells during metaplasia. We confirm that scattered cleaved-caspase-positive cells can be seen. The overall rate is about the same under all conditions (WT, Rapamycin treated, and *Gnptab$^{-/-}$* tissue), which may explain why there is approximately the same tissue mass at d5 post cerulein in each cohort of mice, whereas there is clear chief cell dropout in the stomach when lysosomes/autophagy is disrupted.

***Revision in Manuscript**: We have included these results as an additional supplemental figure (Appendix Fig. S4) with representative images of all analysis stages and experimental conditions stained with cleaved caspase. We discuss in the Results the increased cell death in *Gnptab$^{-/-}$* unit bases in the stomach with HD TAM Day3 as further support for the fact that chief cells that cannot undergo autodegradation in paligenosis either remain trapped in a differentiated state or undergo apoptosis.

3) "Fig3A. The authors should label the fluorescence more clearly in each image. It is also not clear what the arrow denotes in the image 3. "

***Response and Revision**: Agreed, revised.

4) "The authors propose that the initial inhibition of mTORC1 upon injury triggers autophagy, but the evidence seems missing. The authors could test this notion by using a mTOR activator just before injury induction and see if it could prevent autophagy and subsequent reprogramming."

***Response and Revision:** We agree we do not have any direct evidence for this assertion. Barring complex genetic intervention, we have been unable to find a method for activating mTORC1 in vivo using a drug or by increasing amino acids in the diet – we are in process of developing in vitro cell systems as a future direction. We have change our language in our Results and Discussion accordingly. If it will assuage the editors and Referee, we will state that we have another ongoing study in mice null for a genetic attenuator of mTORC1. The preliminary results from this study support that inability to deactivate mTORC1 does inhibit reprogramming.

5) "Fig5. The authors should add time-course (e.g. from day 1 to day 5) quantitative data for lysososome or LC3-GFP for pancreas to show the kinetics of autophagy during ADM."

***Response and Revision**: As the Referee stated early, it has been shown previously in the pancreas that autophagy and lysosome are activated *en route* to dedifferentiation, whereas the stomach findings are novel. Thus, we had not wanted to extensively recapitulate the extant pancreas literature. However, per Referee suggestion, we have conducted an analysis of lysosome dynamics using LAMP1 across various stages of caerulein injury. Our data show an equivalent pattern to that seen in the stomach. The pattern is obvious at a qualitative level. These results are depicted in a new supplemental figure (Appendix Fig. S2).

6) "Suppl. Fig6B. The authors should characterise the acinar remnants in GNPTAB-/- mice 2 weeks post caerulein treatment better (Mist1, SOX9, Krt19 and Amy stainings) as the acinar cells look strikingly different compared with the 5d time point. At 2 weeks, acinar cells display an atypical morphology, which raises questions about their functionality. To see if this is relevant to the mice, the authors could add data on exocrine insufficiency (stool analysis) and body weight."

***Response and Revision:** We agree these cells are interesting, though an extensive analysis seems unwarranted for the current paper, as we agree with the reviewer that these cells are likely quite dysfunctional, as would be expected if they never go through the regeneration process. However, we have performed a limited analysis of these cells, which are clearly acinar cell remnants, based on their lobular organization, E-cadherin positivity, and weak Amylase stain. They do not stain for any ADM or ductal markers, which we had already observed in re: SOX9. Now we show that also stain neither for CK8 nor CK18, two other duct/ADM markers. They also have lost the mature acinar nuclear marker GATA4. Overall, thus, they seem to be partially dedifferentiated acinar cells that have never acquired ADM markers, nor proliferated but are likely to be dysfunctional. We have included these results in a new Appendix Figure S3.

2nd Editorial Decision   18th January 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal, and your patience with our response. Your revised study was sent back to the three referees for re-evaluation. As you will see from their comments enclosed below, the referees find that their concerns have been sufficiently addressed and are now broadly in favour of publication.

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 material & methods and formatting as outlined below, which need to be adjusted at re-submission.

Referee #1:

This reviewer considers the rebuttal to be in order and the revised manuscript improved. If the other referees agree, this paper can be accepted for publication.

Referee #2:

I am happy with the revisions, the authors have done a good job and I think this is a very nice study that will be of interest.

Referee #3:

In the revised manuscript, in my view my comments have been addressed and the results support of the main massage of the manuscript. The authors have also added an improved explanation and justification for the introduction of the term 'paligenosis'. All together, I do not have any major criticism.

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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.

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?	In most experiments, qualitative differences in phenotype were expected, such that investigators, blinded to treatment could assess the treatment. These qualitative, blinded, morphological criteria hinder power analysis relative to a set effect size. Thus, we based our sample size on historic studies where quantitative effect sizes occurred, and more accurate power analyses could be performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All animal experiments were validated in at least three independent experiments. This is indicated in our materials and methods.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In all injury models used, there are characteristic patterns of damage (eg, in stomach HD-Tam model, >90% parietal cells die by 3 days). Any animals who don't show the expected level of damage (eg, parietal cell loss) are excluded from our study, regardless of condition or genotype. No other criterion for exclusion was used, and the criterion was applied equally to all mice.
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.	Animals were co-housed littermates and were randomly chosen for treatment, though no specific a priori randomization method was used
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly chosen, but this was not systemitized.
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.	Morphological differences among treatment groups were largely qualitative. To ensure that this was the case, investigators attempted to assign mice to treatment groups in a blinded fashion. If that was successful, criteria were subsequently quantified, though this was not possible to do completely blinded, as treatments were, by definition, obvious enough that they could be discerned by a blinded osberver without quantification
4.b. For animal studies, include a statement about blinding even if no blinding was done	Initial analysis of effects of treatments were performed at the whole tissue section level by observers blinded as to treatment.
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.	For t-test analyses, normal distribution was assumed. For ANOVA analyses, the data in the current study approximated normally distributed data, though this was not specifically tested, as unequal variance is more of a limiting factor for ANOVA. For ANOVA, it was assured first that there were no outliers in terms of largely different variance in any samples. Tests for normalcy and unequal variance were performed routinely as part of the statistical analyses in GraphPad Prism software. Chi-square analysis did not depend on normal distribution.

Is there an estimate of variation within each group of data?	all quantitative data with statistically analysis is reported with standard deviation or SEM (standard error of the mean), where appropriate; ie, the former when only individual replicates are considered, the latter when the mean of means of replicates are taken
Is the variance similar between the groups that are being statistically compared?	Unequal variance was considered, though was not a factor in the samples in the current manuscript. Tests of variance and normalcy were routinely performed in GraphPad Prism statistical analyses.

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).	reported in supplemental table II
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 strains used in this study are reported in the materials and methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments using animals followed protocols were approved by the Washington University School of Medicine Animal Studies Committee.
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.	yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	we have identified the committees that approved our use of human tissues.
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.	imformed consent was obtained from all human subjects.
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	microarray datasets were publicly deposited and accession numbers noted in material and methods.
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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