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ATF3 induces RAB7 to govern autodegradation in paligenosis, a conserved cell plasticity program

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1st Editorial Decision 8th Oct 2020

Dear Jason,

Thank you for the transfer of your manuscript and the associated referee reports from The EMBO Journal to EMBO reports. Given the potential interest of your findings and the constructive reports from the referees, we would like to offer you to revise your study for potential publication in EMBO reports.

I have meanwhile read your proposed revision plan and suggest to revise your study along the lines you proposed. I feel that it will be important to address the concern whether Rab7b is the only relevant target downstream of Atf3, which can be addressed using the GeneChips you mention and by textual changes and extended discussion. I also feel that a limited set of experiment to address the role of Rab7b in ATf3-mediated lysosome rearrangement would be helpful. I agree that an in vivo context of paligenosis would be more informative but I also agree that such experiments are out of the scope of the current study. Please also address all concerns from referee 3 regarding the analysis of autophagy. It will not be necessary to delineate the mechanism by which Rab7b induces autophagy in this context (referee 3, point 6). Regarding the clinical data: I agree with referee 2 that the current data do not provide strong evidence for a clinical relevance and suggest to either tone down the conclusions or to provide further data (as suggested).

Taken together, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the 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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the

data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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.

Kind regards,
Martina

Martina Rembold, PhD
Editor
EMBO reports

Dear Reviewers and Editors at *EMBO J* and *EMBO Reports*,

Thank you for the thorough review of our manuscript entitled " ATF3 induces RAB7 to govern autodegradation in paligenosis, a conserved cell plasticity program". We appreciate all the thoughtful comments and feel we have addressed each suggestion and critique carefully to improve the manuscript.

There has been a recent reignition of the field of cell plasticity, resulting in numerous publications over the last few years and the introduction of a new Keystone Symposia Series. We have been working hard to better understand the stages of paligenosis, the first defined mechanism that differentiated cells employ to become progenitor-like and aid in injury repair. We have also recently reported that loss of a paligenosis gene can lead to increased tumorigenesis, making our study and careful characterization of paligenosis important to both the cell plasticity and cancer biology fields. The study presented in this manuscript identifies ATF3 as the earliest activated paligenosis gene. We also demonstrate a new mechanism whereby ATF3 transcriptionally upregulates *Rab7b* to induce cell degradation pathways, which is important in cell biology since there are very few descriptions of how Rab GTPases can be transcriptionally controlled. We are confident that our new findings based on the comments and suggestions from the reviewers substantially improve the impact of our manuscript. We hope that the manuscript will now be acceptable for publication in *EMBO Reports*.

We detail our responses and revisions below; however, we would like to highlight the two principal remaining issues that we have addressed first:

1) Reviewers wanted us to clarify the role of autophagy and lysosomes in paligenosis. Though detailed dissection of autophagic flux isn't feasible in whole animals, we have performed wholly new studies to drive home the critical role autophagy plays in paligenosis. We now include **a new experiment** in which we administer hydroxychlororquine (HCQ) in our stomach injury model to reduce autophagy and lysosomal activity. Hydroxychloroquine can be used *in vivo* to block the fusion of autophagosomes with lysosomes. We find that HCQ reduces the number of stomach units that exhibit SPEM after injury with high dose tamoxifen, which indicates that there has been a defect in paligenosis. We also notice that gastric zymogenic chief cells still maintain much of their exocrine granules that are normally degraded in paligenosis Stage 1. This supports that functional lysosomes and autophagolysosomes are required for paligenosis and is consistent with our previous studies with mice lacking *Gnptab* (N-Acetylglucosamine-1-Phosphate Transferase Subunits Alpha and Beta) a gene required for hydrolase trafficking to the lysosome. In addition, when we have previously interrogated paligenosis in mice with mutant autophagy genes (*Atg16L1*, *Atg5* loss, *Atg7* loss), we have not seen any appreciable phenotypes, suggesting that lysosomal function and establishment of autophagolysosomes are likely the critical aspects of autodegradative pathways cells use to execute Stage 1 of paligenosis.

2) A deeper characterization of the relationship between ATF3, Rab7b, and degradation was brought up by several reviewers and we agree that this is an important addition to the manuscript. We include **experiments with two additional RAB7B constructs** in AGS cells to better describe this relationship. We include RAB7B overexpression in *ATF3* knockdown lines to determine if RAB7B is sufficient to drive formation of the large degradation vesicles we have observed *in vivo* and *in vitro*, and find that vesicles are indeed present, even when *ATF3* is knocked down. We also include a RAB7B dominant-negative mutant ($RAB7B^{T22N}$) that disrupts

GTPase activity to define if a functional RAB7B protein is necessary for vesicle formation. We find that in the dominant-negative mutant, there are no degradation vesicles, but rather RAB7B-puncta, indicating that vesicle formation is a process driven by RAB7B's GTPase function and not just RAB7B localization or presence.

RESPONSE TO EDITORS AND REVIEWERS:

Editor's Comments: *"Thank you for the transfer of your manuscript and the associated referee reports from The EMBO Journal to EMBO reports. Given the potential interest of your findings and the constructive reports from the referees, we would like to offer you to revise your study for potential publication in EMBO reports.*

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Response and Revisions in Manuscript: We summarize key additional experiments and the corresponding changes to the manuscript to reflect these new data:

1) As we express in the manuscript, ATF3 likely has more than one target that is important during paligenosis. It had already been published that ATF3 represses *Mist1* (differentiation gene and secretory cell scaling factor) and induces *Sox9* (plasticity gene also associated with cell cycle re-entry). There are also some published studies (mentioned in lines 387-391 of the new manuscript) whose data, when we reanalyzed them, suggest ATF3 may regulate expression of some autophagy genes like *Beclin*-1. With new *in vitro* experiments, we include in Figure 3 of the manuscript, we show that in the case of degradation and Stage 1 of paligenosis that *Rab7b* is key, as overexpression of RAB7B in cells with *ATF3* knockdown exhibit large vesicles phenocopying what we see *in vivo*. While we cannot rule out that *Rab7a* in Stage 1 of paligenosis, we see ATF3 induction of *Rab7b* only.

2) To better assess the importance of autodegradative cell activity in paligenosis, we include the aforementioned hydroxychloroquine experiment in a full supplemental figure (Figure EV2 in the revised manuscript) that shows a key role for lysosomes and/or autophagolysosomes. When *Atf3* is lost, we see a decrease in both lysosomes and autophagosomes and/or autophagolysosomes. We now clarify in the text our evidence for the specificimportance of lysosomes in paligenosis We will include a point-by-point response to all concerns from Referee 3.

3) We include both a revised clean version of the manuscript as well as a redlined manuscript highlighting substantial amendments that improve the manuscript, as guided by the editors and reviewers.

Referee #1: "*This study seeks to investigate the function of ATF3 during paligenotic injury. The manuscript presents data to demonstrate the the role of ATF3/RAB7 axis in three stages of paligenosis. However, there are concerns:*

1) In figure 1E, it is important to show the percentage of electron dense-cargo or organelles surrounded by single membrane versus double membrane. The authors have argued later in Figure 3C that absence of ATF3 reduced the formation of autophagic puncta. Thus, it is important to demonstrate how much paligenotic injury affects autophagosome formation (marked by double-membrane bound vesicles)."

Response:

We thank the reviewer for their careful assessment of our manuscript, especially our characterization of autodegradative events early in paligenosis. This manuscript further delineates how important Stage 1 of paligenosis is and expands on our prior publications regarding this step including the original paligenosis publication in *EMBO J* in 2018. Willet *et al* addressed mechanistic detail into the role of lysosomes and autophagy in paligenosis, including characterization of paligenosis in a mouse with aberrant lysosomal hydrolase trafficking (*Gnptab*-null), so we did not want to duplicate previously published work. Also, we have looked at mice conditionally null or hypomorphic for genes encoding canonical autophagy pathway components (namely, ATG16L1, ATG5 and ATG7) in paligenosis, but so far, we have not found canonical autophagy to be required. These data were not published; however, in this manuscript we now include new experiments with HCQ, the results of which further point specifically to lysosomal function and lysosome-autophagosome fusion as the key steps critical for paligenosis (Figure EV2). We also clarify in the text the importance of lysosomes. We hope the reviewer will allow that dissection of the exact biochemical nature of the requirement for lysosomes is difficult to do wholly within a subset of cells in a living tissue during a narrow time window. The current manuscript focuses not on lysosomes and autophagy but on the ATF3-RAB7B pathway upstream of autodegradative processes. Though exactly how autophagy and lysosomes function in paligenosis is out of the current manuscript scope, we would speculate that likely a non-canonical, organelle-directed autophagy (eg zymophagy) may be activated to generate the autophagolysosomes we observe.

"*2) In Figure 1G-H, it is not clear why the difference in autodegradation count is more striking than acinar cell granule count.*

Response and Revision:

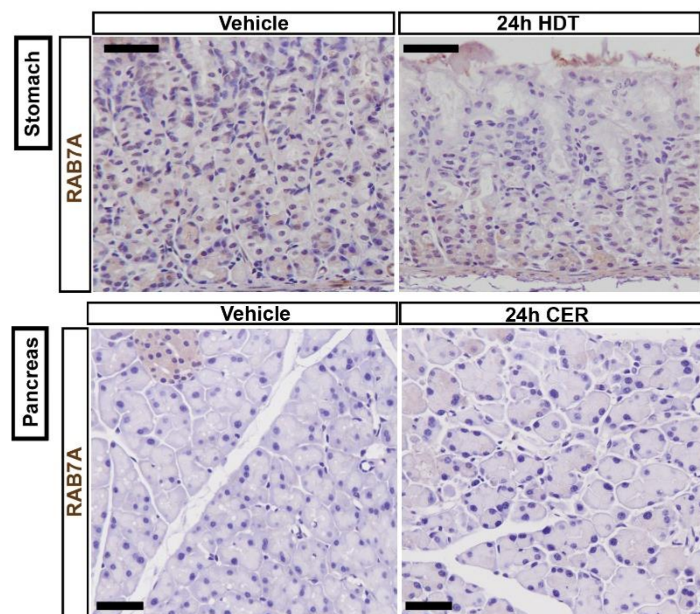
This is a great point that we overlooked in the original manuscript. The cerulein injury model essentially causes cells to degrade themselves by hypersecretion of exocrine granules. Thus, cells are forced to rapidly make more granules and secrete them, so we would argue even a minimal decrease in the granule count in the already-hypersecretory acinar cells is still noteworthy, especially since we find granules located within lysosomes and autophagosomes.

“3) In Figure 1I, it is important to show also the expression of Rab7b at the protein level. Also, it would be helpful to show the localization of Rab7a throughout the injury induction process by immunofluorescent staining

“4) In Figure 1, the authors only showed the expression change of Rab7b in the stomach, but it is not clear how it changes in pancreas acinar cells..”

Response and Revision:

We appreciate this feedback and agree how important it is to show the differences between RAB7A and RAB7B, as they can share similar functions. We hope the reviewer will allow there is some challenge to doing this in the subset of cells that are paligenotic in vivo, especially given the notorious difficulty in detecting RABs of any species by immunostaining. Nevertheless, in the revised manuscript, we have added a wholly new supplementary figure (in the revised manuscript as Figure EV3) that includes Western blots of RAB7, RAB7A, and RAB7B in the stomach and pancreas after injury. We have also performed immunohistochemistry for RAB7A; however, in our hands and in fixed, paraffin-embedded tissue it does not appear to show specific staining (as shown here).



The antibody that recognizes both RAB7 species is the only one we have found to render a specific signal in our tissue. We are confident in specificity of this antibody and in its ability to detect the changes in RAB7B that occur in paligenosis, because we now show that it detects the same type of ballooning, endolysosome vesicular structures in paligenotic cells in two mouse organs AND in human gastric cells in vitro (Revised manuscript Figure 2A,D and Figure 3C). Moreover, RAB7 antibody detection of those structures in vitro is increased by

overexpression specifically of *RAB7B* or *ATF3* mRNA and decreased by knockdown of those same transcripts (Figure 3C,D). Finally, western blot for specifically RAB7B shows RAB7B increases markedly in abundance in both pancreas and stomach from essentially non-detectable levels over the same timecourse as we see in massive increases in RAB7 in paligenotic cells. Western blots across whole tissue for total RAB7 or RAB7A do not show the same consistent synchrony with paligenosis. Thus, all available evidence favors the interpretation that a substantial proportion of the RAB7 signal organized around large vesicles we see in the ATF3-expressing, paligenotic cells in Stage 1 is due to RAB7B.

Therefore, while we cannot specifically rule out RAB7A function in Stage 1 of paligenosis, we are now more certain that RAB7B is critical and that ATF3 transcriptionally regulates *Rab7b.*

"5) In Figure 2B-C, the expression change time courses of ATF3 at mRNA and protein level are inconsistent. Figure 2B shows ATF3 mRNA level peaks at 24h, whereas the protein level peaks early at 6-12h. The authors should show ATF3 qPCR results at 6h and 12h HDT as well.

Response and Revision:

We thank the reviewer for bringing to our attention that we should have included timepoints earlier than 24h in the original manuscript. We now include *Atf3* qPCR expression at 6h HDT, which shows the highest expression level, being consistent with our ATF3 protein expression in the stomach (Revised manuscript Figure 2B). We also include an additional Western blot of ATF3 expression in the cerulein pancreas injury model (now in Figure 2C) to show that ATF3 is expressed early after injury in the pancreas as well.

"6) In Figure 2E, the ChIP result is only shown in stomach tissue. It is not clear whether/how ATF3 induces Rab7b in pancreas."

Response:

In the original manuscript, we inadvertently did not include the supplemental figure that showed ATF3 ChIP-seq in the pancreas, as we duplicated Fig. 2 instead. We now assure that Figure EV4 contains the correct file and thank the reviewer for pointing out our mistake, as this figure is needed to appreciate ATF3 transcriptional regulation of *Rab7b* in the pancreas after injury. Also, the reviewer may note that our analysis of Chip-Seq from a previous study also shows ATF3 binding *Rab7b* in pancreas.

"7) In Figure 4, the authors should show how metaplastic/progenitor gene expression changse in injured ATF3-/- tissue."

Response:

We agree that additional data showing how loss of *Atf3* regulates expression of metaplastic markers will strengthen our claim that ATF3 is required for metaplasia. In Figure EV5 of the revised manuscript we include an additional SPEM marker, CD44v, that is only expressed in metaplasia. We find that without *Atf3*, cells fail to express CD44v even with high dose tamoxifen injury.

"8) In Figure 4, it would also be helpful to show how apoptosis and cell cycle entry events are affected in human cell line with ATF3 knockdown or overexpression."

Response:

The loss of *Atf3 in vivo* results in striking cell death phenotypes after injury and we agree that this would be interesting to look at *in vitro*. As it stands, there is no *in vitro* paligenosis model to be able to conduct an experiment like the one this reviewer suggests. However, the cell lines we use are stable lines and do not exhibit any appreciable changes in cell growth or cell death. Incidentally, our group has two forthcoming reviews on paligenosis and cancer in which we speculate both cancer and tissue culture in general select for perpetually growing cells, which would be exactly the wrong scenario for study of a cell program specifically designed to take large, physiologically — but not mitotically — active cells and convert them into leaner, proliferating progenitors.

Referee #2: *"In this work, authors demonstrate the relevance of atf3 in the transcriptional activation of rab7b that in turn is important for the process of paligenosis. First, I have to admit that I'm not an expert in the field and maybe this explains, at least in part, that I consider the findings novel and relevant. For the same reason, I do not feel comfortable giving a rotund evaluation of the work and the other reviewers will give a more substantiated opinion about the relevance of the findings.*

Technically, I found the experiments sound and my main worry is about the conclusion that changes in proliferation and endosomal/lysosomal activity following atf3 modulation (up or down) are primarily associated with defective paligenosis (other unrelated/independent mechanisms may explain these observations). To my view, this idea should be reinforced by:

1-Showing that the defects imposed by atf3 deficiency are restricted to the differentiated cell population identified by markers and absent from undifferentiated progenitors lacking atf3."

Response:

We thank the reviewer for their thoughtful assessment of our work and appreciate their positive comments about the novelty of our study. The reviewer brings up the question of ATF3 expression and function in differentiated vs. progenitor populations. We are using a global *Atf3* knockout mouse, so stem and progenitor cells that proliferate without paligenosis would also lack *Atf3*. Our phenotypes are observed only in differentiated cells which are the cells we see that markedly induce ATF3 after stomach and pancreas injury. In addition, we performed studies in the pancreas, which lacks resident stem cells after embryogenesis, thus all repair after injury must come from the differentiated cell population through paligenosis. We clarify that our *Atf3*$^{-/-}$ mice lack *Atf3* expression in both progenitor and differentiated cells and reiterate that ATF3 induction is only seen after injury in the differentiated cells in the stomach and pancreas (lines 238-243 in the revised manuscript).

"2-Including some rescue experiments showing the relevance of Rab7b regulation by atf3 in the defective paligenosis found in atf3 defective cells.

Response and Revision:

We thank the reviewer for suggesting this lucrative experiment, as this was the jumping off point for the additional experiments in cell lines presented in Figure 3. We now show that *RAB7B* overexpression is sufficient to induce vesicles for degradation even when *ATF3* is knocked down. We also show that the GTPase function of RAB7B is necessary for vesicle formation.

“3-Demonstrating that the effects of atf3 deletion are different when induced in the differentiated or stem/progenitor compartments of the stomach (or other tissues).”

Response:

Again, we better clarify ATF3 function in stem/progenitor vs. differentiated cell in the revised manuscript. We also reiterate that the adult stomach has a resident stem cell, but the adult pancreas does not. This helps us ascertain that ATF3 is needed, even in tissues that have a stem cell, to promote paligenosis and tissue repair after a severe enough injury.

“4- Finally, the section related with figure 5 is very heterogeneous and, to me, does not contain robust information about how common this atf3-rab7b axis is, or in which processes it is really relevant. In my opinion, the prevalence of atf3-rab7b and paligenosis should be tested in a number of human pre-neoplasic lesions to support the idea that this mechanism is relevant as a process leading to cancer and could be used for therapeutic purposes. Thus, I would encourage the authors to collaborate with clinicians for this specific analysis to further substantiate the clinical relevance of their findings.”

Response and Revision:

We thank the reviewer for pointing out that our ATF3 analysis in humans reads as cursory, but it is based on over 500 human gastric metaplasia samples. We also explain in the discussion (lines 347-351 in the revised manuscript) that ATF3 is present in the nuclei in 11% of the 500 samples we have screened. This is consistent with ATF3 indeed being a (transient) marker of SPEM. We also note that ATF3 is specifically a marker of non-proliferative SPEM, which is expected as we see its expression peak at a specific, transitional time pointwhen mTORC1 expression is decreased (Stage 1 of paligenosis). This dataset is part of an ongoing translational paper in progress, so we hope the reviewer and editors will allow us to reserve all these clinical-translational univariate and multivariate analyses for a future manuscript in which we investigate in detail ATF3 with regard to prognosis, co-morbidity and co-expression with other biomarkers in this large dataset. We still thought a glimpse of the human results would be of interest to readers of the current manuscript. We share two cores from the tissue microarray we are using below with reviewers. Note the scattered positivity for ATF3 in basal cells with SPEM or hybrid SPEM phenotype. Also, we point out that the senior author of the study is a human anatomic pathologist who still attends on service.

Figure for reviewers removed

We also agree that it was unclear how all the elements of the original figure fit together. In the revised manuscript, we place our model of ATF3 and RAB7B by itself in Figure 7. We also move the upset plot identifying candidate paligenosis genes to a supplemental figure (now Figure EV8 in the revised manuscript).

Referee #3:*"In this article Radyk et al., show a role of ATF3 and RAB7 in autophagy during paligenosis. They show that Activating Transcription Factor 3 (ATF3) is induced early during paligenosis and activates the key lysosomal trafficking gene Rab7b. ATF3 and RAB7B are upregulated in gastric and pancreatic digestive-enzyme-secreting cells at the onset of paligenosis Stage 1, when cells induce autophagic and lysosomal machinery to dismantle differentiated cell morphological features. They show that Atf3-/- mice fail to induce RAB7-positive autophagic and lysosomal vesicles, causing increased death of cells en route to Stage 3. They also observe that ATF3 is expressed in human gastric metaplasia and during paligenotic injury across multiple other organs and species."*

Response: We thank Referee #3 for critical review of our manuscript and appreciate their in-depth understanding of our study. We also thank them for the suggestions that have helped make this manuscript stronger.

"Major comments:

1) Figure 1B: Please provide some co-staining with LC3 and quantify autophagy."

Response:

In the revised manuscript, Figure 4 includes LC3 staining in both Wildtype and *Atf3*-null mice before and during paligenosis. We also reference our prior publication, Willet *et al.*, 2018 that

thoroughly characterizes autophagy and lysosomes during paligenosis (including LC3-costaining).

“2) Please provide qPCR analysis of expression of several autophagy genes in Atf3-/- mice.

Response:

First, please see above for the discussion of the requirement in paligenosis for lysosomal function, not necessarily canonical autophagy. However, we agree with the reviewer that it would seem likely that autophagy genes would be increased at the transcript level in paligenosis; however numerous transcriptomic experiments have not supported this model. Rather, the massive changes in lysosomal and autophagic structures seem to be more mediated by changes in flux and in membrane trafficking. This is why RABs are so important: by increasing abundance of specific RABs, a cell can increase relative importance of the membrane compartments those RABs govern. This topic is beyond the scope of the current manuscript, and we can only touch on this topic here, but our lab has continually been surprised about how these large secretory cells scale up during differentiation and down during paligenosis. We have several papers on the scaling up, showing that it is largely affected by increasing the secretory compartment and decreasing lysosomes via RAB3D, RAB26 and by other core membrane trafficking machinery like the endosome-defining ubiquitin ligase MIB1. In a recent study (about to be submitted), we show a critical role for the novel mannose-6-phosphate receptor protein ELAPOR1 in scaling the secretory apparatus. In short, neither scaling up nor scaling down secretory function involves transcriptional regulation of genes encoding specific lysosomal cargo or core autophagy genes or even the digestive enzymes these cells secrete. Within the context of the cell’s scaling (ie the autodegradative aspects of paligenosis vs. the stress response or the downstream reprogramming), the cells transcriptional focus is on membrane trafficking, not autophagy ubiquitination or lysosomal hydrolases. We thank the reviewer for the opportunity to digress a bit to share and speculate on this fascinating topic of the transcriptional regulation of cell scaling in vitro!

“3) Figure EV2 A, B C cannot be found (it is the same with Figure 2). Therefore, there are no data supporting ATF3 transcriptional role.”

Response:

We thank the reviewer for pointing out our clerical mistake in attaching the wrong file for the supplemental figure. We now assure that the new Figure EV4 contains the correct file and proper assessment of ATF3 transcriptional regulation of *Rab7b* in the pancreas can now be made.

“4) Please show evidence that RAB7 antibody used for immunofluorescence and immunocytochemistry specifically recognise RAB7B and not RAB7A.”

Response:

We refer the reviewer to the above discussion on this important point that we hope to have clarified to the extent available reagents have allowed us.

“5) Do autophagy mutants have a defect in paligenosis?”

Response:

Please see above where we address this important point in some depth.

"6) How Rab7 activates autophagy in the context of paligenosis?"

Response:

The literature shows that RAB7B can interact with ATG4B, a cystine protease required for autophagy, so this might be one potential mechanism used during paligenosis (Kjos *et al*., 2017). However, we believe a more likely mechanism for how RAB7B is functioning to increase degradation in paligenosis is likely through Cathepsin D, since RAB7B mutations impair CTSD maturation (Progida *et al*., 2020).

"7) Please provide more images with apoptotic cells in figure EV5."

Response and Revision:

Thank you for the suggestion. We now add an additional panel showing Cleaved CASP3 expression following 72h HDT in Wildtype and *Atf3*-null mice. This is now Figure EV7 in the revised manuscript.

"8) Please provide quantification in Figure EV6."

Response:

The reviewer points to the Axolotl limb regeneration figure, which shows the fraction of cells expressing paligenosis genes ATF3, IFRD1, and RAB7B following limb amputation. This is our analysis of data from a published single-cell RNA sequencing dataset (Gerber *et al*., 2018) and cannot be quantified in the manner the reviewer suggests. However, we clarify the usefulness of this experiment in the text of the revised manuscript.

"Minor comments:

1) Please provide some better images in Figure 2F ATF3/CSTD staining"

Response:

We thank the reviewer for this suggestion. In the revised manuscript, Figure 4 includes LC3 staining in both Wildtype and *Atf3*-null mice before and during paligenosis. We also note reference our prior publication, Willet *et al*., 2018 that thoroughly characterizes autophagy and lysosomes during paligenosis (including LC3-costaining).

"2) Rab7a sometimes is written Rab7. Please correct

3) In the introduction some information is needed about RAB7A and RAB7B and what are their different functions/ how similar or different they are."

Response:

We thank the reviewer for pointing out the notation we used throughout the paper and suggesting an expanded introduction on RAB7A/RAB7B. In the revised manuscript Introduction, lines 72-86, we detail the similarities and differences in function of RAB7A and RAB7B.

1st Revision - Editorial Decision 2nd Jun 2021

Dear Jason,

Thank you for the submission of your revised manuscript to EMBO reports. I am sorry for the delay in handling your manuscript. Unfortunately, referee 3 was not available anymore but we have received the reports from referee 1 and 2, who both support publication of your manuscript. Referee 2 indicates that inclusion of clinical data could have improved the relevance of the manuscript but since we explicitly offered the option to tone down the conclusions regarding clinical relevance instead of providing further data, this does not preclude publication. Please ensure to describe the clinical relevance based on the current data in the most appropriate manner.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

1) Please reduce the number of keywords to 5.

2) The paragraph Methods needs correcting to Materials and Methods.

3) Please re-order the manuscript sections according to the order listed in our guide to authors. See also https://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

4) Figure callouts:
- The callout to Fig 6A should be for Fig 6 only as there is only one panel.
- Fig EV1, EV2 + EV7 panels are not called out.
- Fig EV5B+C panel callouts are missing.
- The Reagents table has no callout.

5) Movies: Please remove the legends from the word text file and provide each legend as individual README.txt file. Then zip the movie together with its legend and upload the .zip file.

6) Please note that we can only typeset up to 5 EV figures. Your manuscript currently contains 8 EV figures. Please either reduce their number by combining several of the figures or alternatively, shift some of the figures to an Appendix pdf. If you choose the latter option, please adhere the following format: The figures are called Appendix Figure Sx, the Appendix is a single pdf including all figures and their legends and it needs a title page with a table of content and page numbers.

7) Please upload the two EV tables as separate files.

8) Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see the required changes in the attached Word file. Please note that we have added the EV figure legends to the main manuscript. These must be part of the main manuscript file as a separate section after the main figure legends.

9) In addition, I note the following points regarding statistics:
The data in Fig 1D/E and Fig 4E were obtained from two mice only and the statistical comparison therefore rests on n=2 and should be removed.
The number and the nature of the replicates in Fig 1G/H is unclear. Please clarify these in the figure legend and please do not provide p-values in case the data are based on 2 mice.

10) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. I note that Gerber et al, 2018 and Miao et al., 2020b could be cited as Data references.
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
<https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

11) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The revised manuscript is strengthened greatly and all concerns have been addressed.

Referee #2:

Although I'm not an expert in palinogenesis (or maybe because of this) I think. that authors have substantially improved the manuscript and addressed most of my concerns. About the importance of including clinical data I still find it would improve the relevance of the manuscript but I trust in the criteria of the other reviewers and the editor.

2nd Authors' Response to Reviewers 4th Jun 2021

The authors have addressed all editorial requests.

2nd Revision - Editorial Decision 18th Jun 2021

Dr. Jason Mills
Washington University
Gastroenterology
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4940 Parkview Place
St. Louis, Missouri 63110
United States

Dear Jason,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Jason C Mills
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51806V2

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

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

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

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

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

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

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

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

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Statistical justification for animal numbers is based on our previous experience, consultation with biostatisticians, and using the sampling power analysis approach
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least the minimum numbers of animals are used to achieve statistical significance in any determinations.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Yes, samples and animals were randomized when allocating into each treatment groups. Experimental cohorts were randomly designated at birth for specific animals to minimize bias.
For animal studies, include a statement about randomization even if no randomization was used.	Animals (that were age-matched and littermates) for each experiment were randomly selected to belong to a treatment or control group through simple randomization.
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.	Measurements were blinded to prevent the introduction of experimental bias
4.b. For animal studies, include a statement about blinding even if no blinding was done	Statement made in the methods section
5. For every figure, are statistical tests justified as appropriate?	Statistical tests utilized are described in each figure legend and a more thorough explanation of the test are included in the methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	This is detailed in the methods: In cases involving more than two samples to compare, significance was determined using ANOVA with post-hoc correction: Tukey was used to assess statistical significance if multiple conditions were compared; Dunnett post-hoc when comparing multiple samples to a single control. Otherwise, unpaired, two-tailed Student's t-test was used to determine significance. Data that would violate normalcy by study design were analyzed by nonparametric Mann-Whitney U test. Formal tests for normalcy were not performed.
Is there an estimate of variation within each group of data?	Yes, SD or SEM are included in each plot

Is the variance similar between the groups that are being statistically compared?	Yes

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).	All antibodies were either cited or the catalog and clone number were included in the supplemental information
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source of cell lines are included in the supplemental information and cells are routinely tested for mycoplasma.

* 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.	Details are included in the methods: All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Mice were maintained in a specified pathogen-free barrier facility under a 12-hour light cycle. All mice used in experiments were 6–8 weeks old. Male and female mice were used for each experiment and we did not note any differences due to sex. Statistical justification for animal numbers is based on our previous experience, consultation with biostatisticians, and using the sampling power analysis approach.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments involving animals were performed according to protocols 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.	Human gastric pathological tissue specimens were obtained with approval by the Institutional Review Board of Washington University School of Medicine
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.	Informed consent was received from all patients for the use of their tissue and experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report
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.	Samples are from resection and biopsy specimens from standard-of-care surgical and endoscopic procedures at the institution. They are, by their nature, finite.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

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

G- Dual use research of concern

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