EMBO reports

Pseudomonas aeruginosa survives in epithelia by ExoS-mediated inhibition of Autophagy and mTOR

Lang Rao, Indhira De La Rosa, Yi Xu, Youbao Sha, Abhisek Bhattacharya, Michael J Holtzman, Brian Gilbert, and N. Tony Eissa
DOI: 10.15252/embr.202050613

Corresponding author(s): Lang Rao (Lang.Rao@va.gov)

Review Timeline:

Submission Date:	9th Apr 20
Editorial Decision:	29th Apr 20
Revision Received:	28th Sep 20
Editorial Decision:	13th Oct 20
Revision Received:	29th Oct 20
Accepted:	12th Nov 20

Editor: Achim Breiling

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 29th Apr 2020

Dear Dr. Eissa,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

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

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

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

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

When submitting your revised manuscript, we will require:

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs

to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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

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

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

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

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

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

See also: http://embor.embopress.org/authorguide#datadeposition

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

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

Data availability

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

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

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

Moreover, I have these editorial requests:

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

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

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please add a conflict of interest statement and a paragraph describing the author contributions to the manuscript, next to the acknowledgements.

10) Please also provide 5 keywords on the title page.

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.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

This study shows that viability of intracellular P. aeruginosa bacteria is suppressed by autophagy, which is otherwise impacted by ExoS ADPRT at multiple steps. The field is currently lacking comprehensive studies on how P. aeruginosa survives inside of epithelial cells; the findings in this study will be of interest to those studying P. aeruginosa virulence. Identifying specific mechanisms of ExoS effects on host cells is notably difficult due to its broad substrate specificity, however, the authors have clearly shown and explained how, while ExoS blocks the mTOR pathway, autophagy is still downregulated by a yet-unknown mechanism at the formation of autophagosomal

membranes. The manuscript can be strengthened by clearer writing, developing a focus and providing more depth in the discussion section, and clarifying a few experimental results.

Concerns of crucial importance

1. General comment on Western blots: The Western blots might be saturated as presented; it is not clear from the printing. However, the differences in the examined proteins changes in each experiment were usually substantial enough not to raise concern about the authors' conclusions. If the western blots are within a linear range of detection, densitometry and standard deviation of experimental repeats would strengthen their conclusions. Of note, actin is problematic loading control since all three toxins, ExoS, ExoT, and ExoY, interfere with it. While I would not request every experiment using actin as a loading control be repeated, please explain in the methods how equal amounts of protein were loaded onto gels-was this normalized by BCA? Did the authors check total protein by coomassie, or after transfer by Ponceau stain? Generally, despite examples of using actin as a loading control in the literature, P. aeruginosa studies should use housekeeping genes not known to be targeted by the exotoxins, or better, normalize samples by total protein.

2. The writing requires improvement. The manuscript has many grammatical errors mostly involving verb tenses and inconsistency with plural forms of words. In addition, there are several paragraphs that are only 2-3 sentences and do not appear to develop a single idea. The discussion requires substantial revision. It restates the same concepts, and only provides very general statements about the known activity and effects of ExoS toxicity toward host cells, which are more suited to the introduction. The discussion appears to miss the importance of the paper's findings in regard to promoting pathogen survival, and focuses more on speculative drug targets. There are also concerning errors made about the actual data contained in the paper, e.g., stating "mRNA levels were analyzed." More adequate topics for discussion could include probable mechanisms for Vps34 inhibition, or placing these findings in what is currently known about P. aeruginosa survival within host epithelial cells.

3. With regard to Figures 2C and 3C, and others showing autophagosomal puncta by different methods, do the authors know the frequency with which cells are invaded by P. aerguinosa? Generally, studies looking into P. aeruginosa internalization in other epithelial cell lines show that fewer than half of the cells have intracellular bacteria, largely because ExoS and ExoT can reduce bacterial internalization. Is autophagosome or autolysosome formation detected in all host cells, or just those that contain intracellular bacteria? Perhaps this question can be answered by additional data obtained to generate figure 4A.

4. Can authors explain the ExoS band doublet and size differences in figures 6A and 6B? In 6B the inhibition of LC3 II generation is not apparent but it does appear that more LC3 I is present. Is this finding sufficient to support the authors' conclusions?

5. Figure S7: ExoS reduces amount of P-4EBP1 detected, and most of the data is consistent with that with one exception: P-4EBP levels from the ΔST mutant infection in the last column-should there be a band here like in ΔSTY and ΔSY? Can the authors explain this?

6. The model is a bit confusing, as it shows Type three secretion by extracellular bacteria, but not cytoplasmic bacteria. Do the authors intend to suggest anything about whether extracellular bacteria specifically contribute to the fate of intracellular bacteria? Have they considered whether intracellular bacteria also secrete ExoS? In addition, WT P. aeruginosa can be found in host cell cytoplasm and in membrane-blebs, however the subcellular localization of ΔpscD is not known, or addressed in this study. Do the authors suggest it also enters the cytoplasm (Figure 10) This would

be different from ΔexsA (another mutant that does not make the T3SS), which appears to remain in phagosomal compartments according to the literature.

Minor Concerns:
1. Were the CFU counts in 1B normalized the number of bacteria internalized at an earlier timepoint? Please revise the text to in lieu of adding an additional experiment since the conclusion is difficult to make without a reference to invasion levels. This issue for P. aeruginosa was addressed well in Figures 1D and 1E so this concern is relatively minor.

2. Figure S6 should be in the main text.

3. Have the authors considered testing whether Vps34 is a substrate of ExoS-ADPRT? If it has not yet been tested, this may be a good addition to the discussion section.

Referee #2:

The manuscript by Rao et al. is a very comprehensive analysis of the mechanism by which P. aeruginosa ExoS promotes survival of the bacteria in epithelial cells. The authors link survival to inhibition of autophagy. The authors present evidence that this inhibition of autophagy affects survival in a lung model of infection. They link inhibition of autophagy to the ADP-ribosyltransferase activity of ExoS and show that ADP-ribosylation of Ras leads to down-regulation of mTor signaling, while, at the same time, also inhibiting activation of autophagy by preventing activation of the Vps34 PI3K through an unknown mechanism. The work is thorough and constitutes a significant advance for the field. My issues, listed below, are minor.

Minor issues:

P1 There is a listing of 5) contributed equally to this publication, but none of the authors are identified using this superscript

P3, 5th line from the bottom (line numbers would have been great, btw!) - ExoS and ExoT have GTPase activating protein function, not G-protein activating function

P4 top line. The citations aren't quite correct. I don't think that the Vance et al. pub shows anything about survival in airway epithelial cells. The second paper cited from the Fleiszig lab does examine survival in epithelial cells, but not airway epithelial cells. Gerry Pier has done some work with intracellular PA in airway epithelial cells, but I don't know if her examined the role of the T3SS.

If more than two samples are in the experiment (e.g. 2C, 3C, 4B), one-way ANOVA + post-hoc test should be used for comparisons (not Student's T-test).

Fig. 2B. LC3-I seems to shift up a bit in the WT infected cells. Does it get ADP-ribosylated by ExoS?

Fig. 2B the chloroquine-treated cells are presumably from a different set of cells than the lysates in which ExoS/T/Y/actin are being detected (those correspond to the top LC3 blot, right?) It might be good to separate that LC3 blot panel from the other ones to make this point explicitly, i.e. have one block be LC3 (w/o CQ), ExoS, ExoT, ExoY, actin and have the LC3 +CQ panel be distinct from this

block (perhaps below it with a space to the block of blots above)?

Gene names should be italicized

Fig. S2A the lane labels and Western lanes don't quite line up. Also, what happened to the LC3-I band in that blot? (compared to, for example Fig. S2B, or Fig. 2B)

I'm not sure I entirely agree with "Interestingly, A549 cells treated with rapamycin and infected with WT P. aeruginosa still showed a reduced LC3II when compared to non- infected cells (Fig. S2A)" The levels look pretty similar to me. There is some evidence for this reduction in the S2B experiment. I just don't think the LC3 blot in S2A was very good (see above).

In general the blots in this manuscript seem to have been adjusted to increase the contrast. The adjustment often seems to be so strong, that subtle differences (such as the one noted above, or in Fig. 7D below are difficult to discern.

Fig. 4C. Could you please give a bit more detail in the figure legend (what was the MOI, how long did the infection last, what about the gentamicin treatment? Was it 1h as before?)

Fig. S6 results are pretty interesting. Maybe add them into Fig. 5?

Bottom of P12. "Together, this experiment suggested cytotoxin protein ExoS in P. aeruginosa could protect the bacterial from eliminating by autophagy in vivo." Some errors here, how about "Together, these experiments suggest that the P. aeruginosa effector protein ExoS protects the bacteria from being eliminated by autophagy in vivo."

Fig. 6D. The figure legend states that the cells were infected with P. aeruginosa producing ExoS with it's ADPRT activity intact (S A+), but the label above the lanes in Fig. 6D says S G+A-, which suggests that the ADPRT activity is inactivated (note that the figure legend makes sense with the data, so I assume the figure labeling is wrong.

Fig. 7D It looks to me like the P-S6 band isn't greatly reduced here. Another issue with this experiment: isn't ExoS toxic (it induces apoptosis, right?) How did these cells survive for 24h? Given the weak result and issues with the experiment, the authors may want to remove this panel.

Fig. S7 - lane labeling does not line up with lanes on blots.

Ras G12V/R41K is labeled Ras R41K in figures, which is confusing (since a priori, this sounds like the Ras variant only has the R41K mutation). The labeling should be changed (in Fig. 8 and S8)

Figure legend Fig. 9. ** is $p<0.01$?

The Vps34 results seems interesting, but I'm a little fuzzy on the details here. Is Vps34 on its own active, or does it need to be in a complex? Is it usually modified in some way (e.g. phosphorylation) to activate the PI3K? Does ExoS ADP-ribosylate VPS34 directly? (notably, ADP-ribosylation is not always associated with a gel shift, akin to the one seen in Ras, or ExoS itself)

P20 top, "by avoiding" is duplicated

P20 middle, "P. aeruginosa could injects up to four .." "injects" should be "inject"

P20 bottom "acting cytoskeleton" should be "actin cytoskeleton"

P21 bottom "Autophagy pathway involves a cascade of event" should be "The autophagy pathway" and "events"

Referee #3:

This manuscript studied the role of P. aeruginosa T3SS in protecting P. aeruginosa from autophagy elimination by the activity of ADP ribosyl transferase. It is interesting because most publications reported that P. aeruginosa induces autophagy (e.g. doi: 10.1016/j.bbrc.2018.07.071.), but does not inhibit it. This new finding will help people to understand the complex role of T3SS and the interaction between host and P. aeruginosa.

However, the current version of this paper is not sufficient to conclude the conception and experiment design is not highly rigorous as the rationale to focus on epithelial cells especially on A549 is not justified the original discovery of autophagy (ref 18) in macrophages has also touched epithelial cells. Authors may use other epithelial cells and mouse and human alveolar primary cells (they used airway cells instead) to substantiate the notion. Second, it is established that mTOR inhibits autophagy. If ExoS inhibits mTOR, why t3ss knock out induced more autophagy? This does not support the hypothesis and conclusion.

The autophagic flux indicators are not measured and LC3 was extensively used but may not be representative for autophagosomes. Some of the imaging data are not in high quality and convincing. Although they did work in vivo the animals appear not based on epithelial KO in epithelial cells but in airway ciliated cells (FOXJ1-Cre). Additional assays are required to present appropriate and clear evidence to support the results and the conclusion. They also used several statistic methods in figures but not discussed in methods and missed detail about animal age, sex, group size (reproducibility should be considered and discussed in each of figures), etc. Hence, this manuscript is not acceptable to be published by EMBO Reports at this time.

Major concern:
1 Figure 1 C-D, the WT P. aeruginosa should be compared with the mutant strain of their differences between response to autophagy and their ability to invade cells by merging C and D as well as add more analysis. A549 is not phagocyte and P. aeruginosa is extracellular bacterium. It should not have much CFU in the lysates.
2 Figure 2A-B, western blotting assay method for bacterial proteins should provide more detail. Usually need 10% TCA for precipitating the proteins.
3 Figure 2A-B again, actin is just the reference protein for host cells (A549), bacterial protein also needs reference protein like the cell wall- associated protein RpoA.
4 When you work with autophagy, not only ILC3 but also whole autophagy flux associated proteins (at least one in each step) should be detected with different assays.
5 Figure 4A , the images are very unclear for quantitation. Please state the counting approach in the figure legends. Image J or quantity one or other methods
6 Pay attention to distinguish the writing between gene and protein. Italics and case.
7 Most of the WB bands are in poor quality, which should be improved.
8 GFP-LC3 dots cannot sufficiently represent autophagosome. It also could include the earlier or

later stage of autophagosome. Hence in figures dealing with LC3 expression or LC3 dots, please specify what is measured, not use autophagosome.
9. Figure 8 and the manuscript title. It is hard to understand that "ExoS ADP-ribosyltransferase activity inhibited mTOR". We know that mTOR inhibits autophagy. Once mTOR is inhibited, autophagy should be induced. If ExoS inhibited mTOR, why t3ss knock out induced more autophagy. Although the negative regulation of mTOR (AMPK and p53 signaling) promotes autophagy, the conclusion may be counterintuitive.

Minor concern:
1 Move figure s5 to major figure will be better.
2 Mouse gene knock out usually use Atg7-/- while not Atg7Δ.
3 keep the consistence of ns: not significant. They are sometime ns sometime NS for now.
4 Scale bar is missing in figure 5B.

Manuscript Number: EMBOR-2020-50613-T
We appreciate the reviewers' comments and we provide point-by-point responses

Reviewer #1

Reviewer: This study shows that viability of intracellular P. aeruginosa bacteria is suppressed by autophagy, which is otherwise impacted by ExoS ADPRT at multiple steps. The field is currently lacking comprehensive studies on how P. aeruginosa survives inside of epithelial cells; the findings in this study will be of interest to those studying P. aeruginosa virulence. Identifying specific mechanisms of ExoS effects on host cells is notably difficult due to its broad substrate specificity, however, the authors have clearly shown and explained how, while ExoS blocks the mTOR pathway, autophagy is still downregulated by a yet-unknown mechanism at the formation of autophagosomal membranes. The manuscript can be strengthened by clearer writing, developing a focus and providing more depth in the discussion section, and clarifying a few experimental results.

Concerns of crucial importance

1. General comment on Western blots: The Western blots might be saturated as presented; it is not clear from the printing. However, the differences in the examined proteins changes in each experiment were usually substantial enough not to raise concern about the authors' conclusions. If the western blots are within a linear range of detection, densitometry and standard deviation of experimental repeats would strengthen their conclusions. Of note, actin is problematic loading control since all three toxins, ExoS, ExoT, and ExoY, interfere with it. While I would not request every experiment using actin as a loading control be repeated, please explain in the methods how equal amounts of protein were loaded onto gels-was this normalized by BCA? Did the authors check total protein by Coomassie, or after transfer by Ponceau stain? Generally, despite examples of using actin as a loading control in the literature, P. aeruginosa studies should use housekeeping genes not known to be targeted by the exotoxins, or better, normalize samples by total protein

Response: According to the reviewer's suggestion, we re-adjusted all the western blots to the respective original backgrounds. For all the immunoblotting experiments, we first measured the total amount of protein by BCA method and then loaded an equal amount of protein (50 μg) in each lane of the gels. In our study, we took a series of steps to exclude the possibility of bacterial proteins interfering with total cellular protein. In the bacterial infection experiment, before harvesting infected cells for SDS-PAGE, we used gentamycin to kill all uninternalized bacteria outside the cells and we removed these bacteria by washing with PBS for 3 times. It should be noted that, unlike phagocytic cells, epithelial cells such only take up a small number of bacteria during pathogen infection. Thus, the amount of bacterial protein is so small compared to the total cellular proteins. The method we are using is a well-established method that has been widely used in other bacterial infection studies (reference (4) and reference (25) in the manuscript). We could not find evidence to suggest that

actin could be modified by ExoS ADP ribosylation directly. Further, several studies of ExoS has utilized actin as a control protein (Sun & Barbieri, 2004; Belyy *et al*, 2016; reference (28) in manuscript)

Reviewer: 2. The writing requires improvement. The manuscript has many grammatical errors mostly involving verb tenses and inconsistency with plural forms of words. In addition, there are several paragraphs that are only 2-3 sentences and do not appear to develop a single idea. The discussion requires substantial revision. It restates the same concepts, and only provides very general statements about the known activity and effects of ExoS toxicity toward host cells, which are more suited to the introduction. The discussion appears to miss the importance of the paper's findings in regard to promoting pathogen survival and focuses more on speculative drug targets. There are also concerning errors made about the actual data contained in the paper, e.g., stating "mRNA levels were analyzed." More adequate topics for discussion could include probable mechanisms for Vps34 inhibition or placing these findings in what is currently known about P. aeruginosa survival within host epithelial cells.

Response: Thanks for the suggestions. We have modified the texts in the revised manuscript. Grammatical and linguistic mistakes have been carefully checked and corrected. We rewrote the discussion section adding more discussion on the potential mechanism of how ExoS influences the activity of the autophagic vps34 kinase complex.

Reviewer: 3. With regard to Figures 2C and 3C, and others showing autophagosomal puncta by different methods, do the authors know the frequency with which cells are invaded by P. aeruginosa? Generally, studies looking into P. aeruginosa internalization in other epithelial cell lines show that fewer than half of the cells have intracellular bacteria, largely because ExoS and ExoT can reduce bacterial internalization. Is autophagosome or autolysosome formation detected in all host cells, or just those that contain intracellular bacteria? Perhaps this question can be answered by additional data obtained to generate figure 4A.

Response: Figure 2C and Figure 3C are the representative immunofluorescence images of GFP-LC3 puncta found in A549 cells when infected by different bacteria. We used the same criteria for puncta quantification in all experimental setups. For each assay more than 100 cells were counted, and 3 independent assays were done for each bacterial infection. In our study, we compared the CFU after infecting A549 cells for 1 hour with wt and PscD mutant (we believe that bacterial elimination by autophagy is negligible in this short time of incubation and the recovered bacterial CFU reflects the internalized bacteria). However, we found no significant difference in CFU between infection by wt and PscD mutant (Figure 1D and 1E). Autophagosome or autolysosome formation can be detected in all host cells, regardless of infection. However, the number of autophagosomes can vary depending on whether the infection upregulates or down-regulates autophagosome formation.

Reviewer: 4. Can authors explain the ExoS band doublet and size differences in figures 6A and 6B? In 6B the inhibition of LC3 II generation is not apparent but it does appear that more LC3 I is present. Is this finding sufficient to support the authors' conclusions?

Response: Thank you for raising this interesting point. Our result supported the previous finding that ExoS can ADP ribosylate itself (Riese et al, 2002). The slightly higher ExoS band resulted from a change in molecular weight caused by ADP ribosylation. As shown in Figure 6A and 6B, only ExoS with ADP ribosylation activity (ExoSwt ExoS G-A+) has increased molecular weight. Similar results were also found in cells expressing ExoS plasmid (Figure 6c). This observation provides evidence showing that the ExoS ADP ribosylation domain works as expected.

Reviewer: 5. Figure S7: ExoS reduces amount of P-4EBP1 detected, and most of the data is consistent with that with one exception: P-4EBP levels from the ΔST mutant infection in the last column-should there be a band here like in ΔSTY and ΔSY? Can the authors explain this?

Response: Thanks for bringing up this question. It seems that *P. aeruginosa* ΔST could also inhibit P-4EBP, suggesting that ExoY probably could affect 4EBP phosphorylation through some unidentified mechanism. Identifying that mechanism is beyond the scope of this manuscript.

Reviewer: 6. The model is a bit confusing, as it shows Type three secretion by extracellular bacteria, but not cytoplasmic bacteria. Do the authors intend to suggest anything about whether extracellular bacteria specifically contribute to the fate of intracellular bacteria? Have they considered whether intracellular bacteria also secrete ExoS? In addition, WT P. aeruginosa can be found in host cell cytoplasm and in membrane-blebs, however the subcellular localization of ΔpscD is not known or addressed in this study. Do the authors suggest it also enters the cytoplasm (Figure 10) This would be different from ΔexsA (another mutant that does not make the T3SS), which appears to remain in phagosomal compartments according to the literature.

Response: We appreciate the reviewer's comment. The model is drawn as is for simplicity purposes. The type III secretion system (T3SS) is able to inject the cytotoxins in the target cell's cytoplasm directly upon contact to cellular membranes. (reference (9) in the manuscript). It is believed that the T3SS's function depending on successfully attachment of the cellular membrane. We agree with the reviewer that it is possible for intracellular bacteria to secrete the toxin ExoS spontaneously, but we do not have direct conclusive evidence to prove it. We think the subcellular localizations of ΔpscD and other *P.aeruginosa* strains are partially located in the cytoplasm in autophagosome and partially located in phagosome las shown in Figure 4C.

Reviewer: Minor Concerns: 1. Were the CFU counts in 1B normalized the number of bacteria internalized at an earlier timepoint? Please revise the text to in lieu of adding an additional experiment since the conclusion is difficult to make without a reference to invasion levels. This issue for P. aeruginosa was addressed well in Figures 1D and 1E so this concern is relatively minor.

Response: We appreciate the reviewer comment. We have revised Figure 1 as suggested by the reviewer.

Reviewer: 2. Figure S6 should be in the main text.
Response: We agree with the reviewer's comment. We have made a new Figure 5 containing graphs originally from Figure S6.

Reviewer: 3. Have the authors considered testing whether Vps34 is a substrate of ExoS-ADPRT? If it has not yet been tested, this may be a good addition to the discussion section.

Response: We appreciate the reviewer's comment. We tested vps34 by mass spectrometry analysis and the results were not conclusive. We have included a discussion of that concept in the revised manuscript.

Reviewer #2

Reviewer: The manuscript by Rao et al. is a very comprehensive analysis of the mechanism by which P. aeruginosa ExoS promotes survival of the bacteria in epithelial cells. The authors link survival to inhibition of autophagy. The authors present evidence that this inhibition of autophagy affects survival in a lung model of infection. They link inhibition of autophagy to the ADP-ribosyltransferase activity of ExoS and show that ADP-ribosylation of Ras leads to down-regulation of mTOR signaling, while, at the same time, also inhibiting activation of autophagy by preventing activation of the Vps34 PI3K through an unknown mechanism. The work is thorough and constitutes a significant advance for the field. My issues, listed below, are minor.

Minor issues:

P1 There is a listing of 5) contributed equally to this publication, but none of the authors are identified using this superscript.

Response: We appreciate the reviewer comment. We have corrected the author list.

Reviewer: P3, 5th line from the bottom (line numbers would have been great, btw!) - ExoS and ExoT have GTPase activating protein function, not G-protein activating function.

Response: We appreciate the reviewer's comment and we have corrected the error.

Reviewer: P4 top line. The citations aren't quite correct. I don't think that the Vance et al. pub shows anything about survival in airway epithelial cells. The second paper

cited from the Fleiszig lab does examine survival in epithelial cells, but not airway epithelial cells. Gerry Pier has done some work with intracellular PA in airway epithelial cells, but I don't know if her examined the role of the T3SS.

Response: We appreciate the reviewer's comment. We removed the citation (Vance RE *et al,*2005). We agree with the reviewer that the second citation does not specify work on airway epithelial cells. We modified accordingly in the revised manuscript.

Reviewer: If more than two samples are in the experiment (e.g. 2C, 3C, 4B), one-way ANOVA + post-hoc test should be used for comparisons (not Student's T-test).

Response: We appreciate the reviewer's suggestion and we have redone all statistical analysis using one-way ANOVA + Dun's posttest and revised the manuscript accordingly.

Reviewer: Fig. 2B. LC3-I seems to shift up a bit in the WT infected cells. Does it get ADP-ribosylated by ExoS?

Response: We appreciate the reviewer's comment. We have checked the LC3-1 ADP ribosylation of LC3-I by mass spectrometry analysis, but our results were not conclusive.

Reviewer: Fig. 2B the chloroquine-treated cells are presumably from a different set of cells than the lysates in which ExoS/T/Y/actin are being detected (those correspond to the top LC3 blot, right?) It might be good to separate that LC3 blot panel from the other ones to make this point explicitly, i.e. have one block be LC3 (w/o CQ), ExoS, ExoT, ExoY, actin and have the LC3 +CQ panel be distinct from this block (perhaps below it with a space to the block of blots above)?

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly.

Reviewer: Gene names should be italicized.

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly.

Reviewer: Fig. S2A the lane labels and Western lanes don't quite line up. Also, what happened to the LC3-I band in that blot? (compared to, for example Fig. S2B, or Fig. 2B).

Response: We appreciate the reviewer's comment. We have repeated the experiment and made a new figure.

Reviewer: I'm not sure I entirely agree with "Interestingly, A549 cells treated with rapamycin and infected with WT P. aeruginosa still showed a reduced LC3II when compared to non- infected cells (Fig. S2A)" The levels look pretty similar to me. There is some evidence for this reduction in the S2B experiment. I just don't think the LC3 blot in S2A was very good (see above).

Response: We appreciate the reviewer's comment. We have repeated the experiment and made a new figure. In the revised manuscript (Figure EV2A) WT P.aeruginosa reduced LC3II formation compared to the ΔpscD mutant, in the presence or absence rapamycin.

Reviewer: In general, the blots in this manuscript seem to have been adjusted to increase the contrast. The adjustment often seems to be so strong, that subtle differences (such as the one noted above, or in Fig. 7D below are difficult to discern.

Response: We appreciate the reviewer's comment and we have re-adjusted the contrast on western blots.

Reviewer: Fig. 4C. Could you please give a bit more detail in the figure legend (what was the MOI, how long did the infection last, what about the gentamicin treatment? Was it 1h as before?).

Response: We appreciate the reviewer's comment and we have revised the manuscript to include the additional details requested.

Reviewer: Fig. S6 results are pretty interesting. Maybe add them into Fig. 5?

Response: We appreciate the reviewer's suggestion and we have added Fig. S6 to Fig. 5.

Reviewer: Bottom of P12. "Together, this experiment suggested cytotoxin protein ExoS in P. aeruginosa could protect the bacterial from eliminating by autophagy in vivo." Some errors here, how about "Together, these experiments suggest that the P. aeruginosa effector protein ExoS protects the bacteria from being eliminated by autophagy in vivo."

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly.

Reviewer: Fig. 6D. The figure legend states that the cells were infected with P. aeruginosa producing ExoS with its ADPRT activity intact (S A+), but the label above the lanes in Fig. 6D says S G+A-, which suggests that the ADPRT activity is inactivated (note that the figure legend makes sense with the data, so I assume the figure labeling is wrong.

Response: We appreciate the reviewer's comment and we have corrected the error.

Reviewer: Fig. 7D It looks to me like the P-S6 band isn't greatly reduced here. Another issue with this experiment: isn't ExoS toxic (it induces apoptosis, right?) How did these cells survive for 24h? Given the weak result and issues with the experiment, the authors may want to remove this panel.

Response: We agree with the reviewer that the ExoS expressing plasmid could induce apoptosis (reference (28) in the manuscript). In our study, we found that

transfection of wt ExoS expressing plasmid resulted in about <15% cell death within 24hours. However, cell death after transfection with plasmids expressing ADP ribosylation-deficient ExoS was significantly reduced (<5%). It is consistent with the notion that the ADP ribosylation domain of ExoS caused cell death as we presented in Figure EV1. We agree with the referee's suggestion to remove Figure 7D and made a new Figure 7.

Reviewer: Fig. S7 - lane labeling does not line up with lanes on blots.

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly.

Reviewer: Ras G12V/R41K is labeled Ras R41K in figures, which is confusing (since a priori, this sounds like the Ras variant only has the R41K mutation). The labeling should be changed (in Fig. 8 and S8).

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly

Reviewer: Figure legend Fig. 9. ** is p<0.01?
Response: Yes, and we have made that notation.

Reviewer: The Vps34 results seems interesting, but I'm a little fuzzy on the details here. Is Vps34 on its own active, or does it need to be in a complex? Is it usually modified in some way (e.g. phosphorylation) to activate the PI3K? Does ExoS ADP-ribosylate VPS34 directly? (notably, ADP-ribosylation is not always associated with a gel shift, akin to the one seen in Ras, or ExoS itself).

Response: We appreciate the reviewer's comment. Type III phosphatidylinositol 3-kinase VPS34 can generate PI (3)P on either endocytic or autophagic vesicle. However, only the vps34 associated with autophagy protein Atg14L is specifically involved in the initiation and development of the autophagy pathway. Autophagy-specific VPS34 complex I is composed of core components (Atg14L,vps34, and beclin1) and regulators (bcl2, rubicon). The interaction between vps34 and the regulators could either augment or suppress the kinase activity of vps34 and influence autophagosome development (Zhong et al., 2009). This interaction between vps34 and its regulator could be affected by phosphorylation. Vps34 could also be directly phosphorylated at T163 andS165 by AMPK kinase, however, ATG14L determines whether the Vps34 complex is activated or inhibited by AMPK (reference (35) in manuscript). We have addressed the possible mechanisms of ExoS affecting VPS34 either directly or indirectly in the revised manuscript.

Reviewer: P20 top, "by avoiding" is duplicated. P20 middle, "P. aeruginosa could injects up to four .." "injects" should be "inject". P20 bottom "acting cytoskeleton" should be "actin cytoskeleton". P21 bottom "Autophagy pathway involves a cascade of event" should be "The autophagy pathway" and "events"

Response: We appreciate the reviewer's comments and we have corrected the errors.

Reviewer #3

Reviewer: This manuscript studied the role of P. aeruginosa T3SS in protecting P. aeruginosa from autophagy elimination by the activity of ADP ribosyl transferase. It is interesting because most publications reported that P. aeruginosa induces autophagy (e.g. doi: 10.1016/j.bbrc.2018.07.071.), but does not inhibit it. This new finding will help people to understand the complex role of T3SS and the interaction between host and P. aeruginosa.

However, the current version of this paper is not sufficient to conclude the conception and experiment design is not highly rigorous as the rationale to focus on epithelial cells especially on A549 is not justified the original discovery of autophagy (ref 18) in macrophages has also touched epithelial cells. Authors may use other epithelial cells and mouse and human alveolar primary cells (they used airway cells instead) to substantiate the notion. Second, it is established that mTOR inhibits autophagy. If ExoS inhibits mTOR, why t3ss knock out induced more autophagy? This does not support the hypothesis and conclusion.

The autophagic flux indicators are not measured and LC3 was extensively used but may not be representative for autophagosomes. Some of the imaging data are not in high quality and convincing. Although they did work in vivo the animals appear not based on epithelial KO in epithelial cells but in airway ciliated cells (FOXJ1-Cre). Additional assays are required to present appropriate and clear evidence to support the results and the conclusion. They also used several statistic methods in figures but not discussed in methods and missed detail about animal age, sex, group size (reproducibility should be considered and discussed in each of figures), etc. Hence, this manuscript is not acceptable to be published by EMBO Reports at this time.

Major concern:

1 Figure 1 C-D, the WT P. aeruginosa should be compared with the mutant strain of their differences between response to autophagy and their ability to invade cells by merging C and D as well as add more analysis. A549 is not phagocyte and P. aeruginosa is extracellular bacterium. It should not have much CFU in the lysates.

Response: We appreciate the reviewer's comments. Our studies began with the interesting finding that wt and t3ss deficient *P.aeruginosa* were eliminated differently in autophagy-deficient cells. We provided comparisons among the different mutant strains in Figure 4D. In figure1C, D we have compared the recovered CFU after 1 hour and found no significant difference between that of the mutant and wt bacteria. We believe that bacterial elimination by autophagy in this short period of incubation is negligible. Thus, our results indicate that the invading ability of mutant and wt bacteria is not significantly different. Because mutant PscD could not inject any toxin, our results also showed that none of the toxins is affecting bacterial entry into epithelial cells. We realize that A549 is not a phagocytic cell. However, airway epithelium is an important part in host defense and could internalize and eliminate pathogens including *P. aeruginosa*. Further, we have used *Klebsiella pneumoniae,* known as

an airway epithelium pathogen, that could be eliminated by airway epithelium cells, to prove the ability of A549 cells in pathogen elimination (Figure 1B). Finally, autophagic flux indicators were measured by evaluating LC3 type II in the presence or absence of the lysosomal inhibitor chloroquine (27).

Reviewer: 2 Figure 2A-B, western blotting assay method for bacterial proteins should provide more detail. Usually need 10% TCA for precipitating the proteins.

Response: We appreciate the reviewer's comment and we have included the methodology details in the revised manuscript.

Reviewer: 3 Figure 2A-B again, actin is just the reference protein for host cells (A549), bacterial protein also needs reference protein like the cell wall- associated protein RpoA.

Response: We appreciate the reviewer's comment. In our study, we took a series of steps to exclude the possibility of bacterial proteins interfering with total cellular protein. In the bacterial infection experiment, before harvesting infected cells for SDS-PAGE, we used gentamycin to kill all uninternalized bacteria outside the cells and we removed these bacteria by washing with PBS for 3 times. It should be noted that, unlike phagocytic cells, epithelial cells such only take up a small number of bacteria during pathogen infection. Thus, the amount of bacterial protein is so small compared to the total cellular proteins. The method we are using is a well-established method that has been widely used in other bacterial infection studies (reference (4) and (25) in the manuscript). Further, several studies of ExoS has utilized actin as a control protein (Sun & Barbieri, 2004; Belyy *et al*, 2016; reference (28) in the manuscript)

Reviewer: 4 When you work with autophagy, not only ILC3 but also whole autophagy flux associated proteins (at least one in each step) should be detected with different assays.

Response: We agree with the reviewer's comment. autophagic flux was measured by evaluating LC3 type II in the presence or absence of the lysosomal inhibitor chloroquine, as per autophagy guidelines consensus recommendations (27). Moreover, we also visualized the formation of autophagosomes by detecting the formation of GFP-LC3 puncta. Furthermore, we have also dissected the initiation stage of autophagy omegasomes by analyzing mCherry-DFCP1 (Figure 9) and checked the activity of the autophagic vps34 kinase complex (Figure 10). Furthermore, to study the maturation steps of the autophagy pathway we checked the autophagosomes and autolysosomes using the mCherry-GFP-LC3 reporter (Figure EV3). We believe that the study entailed a rather detailed analysis of autophagy.

Reviewer: 5 Figure 4A , the images are very unclear for quantitation. Please state the counting approach in the figure legends. Image J or quantity one or other methods.

Response: We appreciate the reviewer's comment and we have included the methodology details in the revised manuscript

Reviewer: 6 Pay attention to distinguish the writing between gene and protein. Italics and case.

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly.

Reviewer: 7 Most of the WB bands are in poor quality, which should be improved.
Response: We appreciate the reviewer's suggestion. We have repeated and replaced most of the western blots. We also readjusted most of the immunoblots in the revised manuscript.
Reviewer: 8 GFP-LC3 dots cannot sufficiently represent autophagosome. It also could include the earlier or later stage of autophagosome. Hence in figures dealing with LC3 expression or LC3 dots, please specify what is measured, not use autophagosome.

Response: We appreciate the reviewer's suggestion and we have followed the proposed suggestion in the revised manuscript.

Reviewer: 9. Figure 8 and the manuscript title. It is hard to understand that "ExoS ADP-ribosyltransferase activity inhibited mTOR". We know that mTOR inhibits autophagy. Once mTOR is inhibited, autophagy should be induced. If ExoS inhibited mTOR, why t3ss knock out induced more autophagy. Although the negative regulation of mTOR (AMPK and p53 signaling) promotes autophagy, the conclusion may be counterintuitive.

Response: We appreciate the reviewer's suggestion and we can clarify. We agree that the conclusion is counterintuitive and that contributed to making our findings both surprising and novel. We have provided detailed discussion of these conclusions in the revised manuscript. Briefly, figure7 A, B, C, and Figure 8 have shown that ExoS containing *P. aeruginosa* caused the downregulation of P-S6 (which is a target of mTOR), thereby showing that the activity of mTOR has been suppressed. Figure 7D has shown that ExoS ADP-ribosyltransferase activity is necessary for mTOR inhibition.
However, there are also mTOR independent pathways that regulate autophagy. For example, cytosolic Ca2+ can regulate autophagy independent of mTOR (Criollo *et al*, 2007). Moreover, the vps34 kinase activity in autophagy initiation complex could directly be inhibited by kinase inhibitor 3-methyladenine (3-MA) and wortmannin. A more recent study showed that the acetylation of vps34 also influences its kinase activity. In our study, ExoS caused inhibition of autophagy by directly influencing autophagic ATG14-vps34 complex activity and such inhibitory effect negated any possible autophagy induction caused by mTOR inhibition.
As to the question of t3ss mutant triggering autophagosomes, our lab (Xu *et al*, 2007) and others have previously shown that LPS from gram-negative bacteria (including *P.aeruginosa*) could induce autophagy by TRL4 signaling pathway. The t3ss mutant *P. aeruginosa* does not have the toxin secretion system and could not

inject the ExoS into the target cells (Figure 2B). Thus, the autophagy pathway was not inhibited.

Reviewer: Minor concern:
1 Move figure s5 to major figure will be better.
2 Mouse gene knock out usually use Atg7-/- while not Atg7Δ.
3 keep the consistence of ns: not significant. They are sometime ns sometime NS for now.
4 Scale bar is missing in figure 5B.

Response: We appreciate the reviewer's suggestion and we have revised the manuscript accordingly.

References

Belyy A, Raoux-Barbot D, Saveanu C, Namane A, Ogryzko V, Worpenberg L, David V, Henriot V, Fellous S, Merrifield C *et al* (2016) Actin activates Pseudomonas aeruginosa ExoY nucleotidyl cyclase toxin and ExoY-like effector domains from MARTX toxins. *Nature communications* 7: 13582

Criollo A, Maiuri MC, Tasdemir E, Vitale I, Fiebig AA, Andrews D, Molgo J, Diaz J, Lavandero S, Harper F *et al* (2007) Regulation of autophagy by the inositol trisphosphate receptor. *Cell death and differentiation* 14: 1029-1039

Diao J, Liu R, Rong Y, Zhao M, Zhang J, Lai Y, Zhou Q, Wilz LM, Li J, Vivona S *et al* (2015) ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. *Nature* 520: 563-566

Riese MJ, Goehring UM, Ehrmantraut ME, Moss J, Barbieri JT, Aktories K, Schmidt G (2002) Auto-ADP-ribosylation of Pseudomonas aeruginosa ExoS. *The Journal of biological chemistry* 277: 12082-12088

Sun J, Barbieri JT (2004) ExoS Rho GTPase-activating protein activity stimulates reorganization of the actin cytoskeleton through Rho GTPase guanine nucleotide disassociation inhibitor. *The Journal of biological chemistry* 279: 42936-42944

Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* 27: 135-144

Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, Heintz N, Yue Z (2009) Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nature cell biology* 11: 468-476

1st Revision - Editorial Decision 13th Oct 2020

Dear Dr. Rao,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referee #2 has remaining concerns or suggestions to improve the study, we ask you to address in a final revised manuscript.

Moreover, I have these editorial requests:

- Please provide a shorter and more comprehensive title (with not more than 100 characters including spaces).

- Please provide the summary written in present tense. Moreover, please shorten this part. It should have not more than 175 words. Finally, please call this paragraph 'abstract'.

- There are presently 11 main figures. We can accommodate up to 8 main figures. Please fuse the present figures to have 8 main figures, maybe also moving parts to the EV figures, if necessary. Please take care that there are not more than 5 EV figures in the end. Please also carefully update the figure callouts.

- Please add a formal "Data Availability section" to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated or deposited').

- The quality of the Western blot images is rather poor. Could these be provided with higher resolution? Please show these as unmodified as possible, matching to the source data (see below).

- As the Western blots are significantly cropped, we ask you to provide the source data for all the blots (main and EV figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure.

- It seems the panels B and D in Fig. 10 have been spliced together from two source images. Please indicate this with a black separating line.

- Please provide the scale bars for all microscopic images in a similar style and similar thickness that is clearly visible. The scale bars should be shown in the lower right corner of the images, without any text next to them indicating the size. Please define the size in the respective figure legend.

- For the figure panels, please label these with capital letters without the point (A not A.).

- There are some greyish structures (boxes, bars) in Figs. 2C and EV5. Can these be removed?

- Please note our reference format:

http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- For all authors, in particular the corresponding author, please enter all the necessary affiliation information into the submission system upon re-submission.

- Please state in the author checklist compliance the ARRIVE guidelines (section D, 10).

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

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 40 words).
- two to three bullet points highlighting the key findings of your study (2 lines).
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

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

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The manuscript by Rao et al has been improved from its initial submission. The authors have addressed reviewer comments sufficiently. I have no additional concerns and support publication of the manuscript at this stage.

Referee #2:

Most of my comments have been addressed, however

1) Something happened in Fig. 2B.The revised figure now has what was formerly the LC3 blot from the sample without Chloroquine labeled as having chloroquine. The former +chloroquine blot is gone. Is this in error? Was the previous file mislabeled?

2) Gene names are still not italicized consistently (e.g. ΔpscD).

3) There's a new issue with Fig. 7D. The S6 blot has 4 lanes stretched to the same width as the other blots, which have 5 lanes.

Referee #3:

Questions are addressed.

Referee #1:

The manuscript by Rao et al has been improved from its initial submission. The authors have addressed reviewer comments sufficiently. I have no additional concerns and support publication of the manuscript at this stage.

Referee #2:

Most of my comments have been addressed, however

1) Something happened in Fig. 2B.The revised figure now has what was formerly the LC3 blot from the sample without Chloroquine labeled as having chloroquine. The former +chloroquine blot is gone. Is this in error? Was the previous file mislabeled?
We apologize for the mislabeling, we put the chloroquine blot back. We made the correction in the new Fig 2.
2) Gene names are still not italicized consistently (e.g. ΔpscD).
We made the change accordingly.
3) There's a new issue with Fig. 7D. The S6 blot has 4 lanes stretched to the same width as the other blots, which have 5 lanes.
We made the correction accordingly.

Referee #3:

Questions are addressed.
Attachments area

2nd Revision - Editorial Decision 12th Nov 2020

Dr. Lang Rao
VA Long Beach Healthcare System
Southern California Institute for Research and Education
5901 E 7th St
Long Beach, CA 90822
United States

Dear Dr. Rao,

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.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to

our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-50613V3 and be addressed to emboreports@wiley.com.

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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Lang Rao
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-50613-T

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 iustified
- 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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Power analysis was used.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size estimates were done based on prior similar studies done using similar procedures.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Once the mice were chosen for the assay no tested animlal was excluted from the analysis.
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.	Mice were randomized. All experiments included controls.
For animal studies, include a statement about randomization even if no randomization was used.	Mouse cages were randomly allocated to each group after balancing of age and sex. In each bacterial inocation experiment the atg7+ and atg7-mice first genotyped and then randomly assigned to each group after balancing of weight, age and sex.
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.	To minimize the subjective bias, the bacteial injection and mice mortality check were conducted by different personnel.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In animal stuies the investigators were not blinded to the group of allocation
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	The antibodies used in this study are described in Table S1. All antibodies were validated by manufacturers. All antibodies used ithewere validated by manufacturers.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The antibodies of ExoS ExoT and ExoT provied by Arne Rietsch were varified in (Journal of bacteriology. 2008;190(8):2726-38). All other antibodies used in the study were commercial avaible and validated by manufactures.

* 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.	All mice used in the study are originated from C57BL/6J.Transgenic mice with Atg7 conditionally knockout in ciliated airway epithelial cell were generated by crossing FOXJ1-Cre mice to Atg7 flox/flox mice. Mice were housed within a specific pathogen free vivarium and used at 4–12 weeks of age.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The Institutional Animal Care and Use Committee of baylor college of medicine approved all animal studies.
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.	All experimental procedure of the laboratory mice complied with the ARRIVE guideline.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	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