EMBO reports

Mitophagy Regulates Integrity of Mitochondria at Synapses and is Critical for Synaptic Maintenance

Qian CAI, Sinsuk Han, Yu Young Jeong, Preethi Sheshadri, and Xiao Su
DOI: 10.15252/embr.201949801

Corresponding author(s): Qian CAI (cai@biology.rutgers.edu)

Review Timeline:

Submission Date:	2nd Dec 19
Editorial Decision:	17th Jan 20
Revision Received:	1st May 20
Editorial Decision:	29th May 20
Revision Received:	1st Jun 20
Accepted:	5th Jun 20

Editor: Martina Rembold

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 17th Jan 2020

Dear Dr. CAI

Thank you for the submission of your research manuscript to our journal. I apologize for my delayed response, which is due to the Chrismtas/New Year break and the work that had accumulated. We have received the full set of referee reports that is copied below.

As you will see, the referees' opinion on the advance provided are divided. Referee 1 points out that many aspects of the current work have been shown before such as the impairment of mitophagy in AD models or the involvement of Snapin in autophagosome transport. On the other hand, we also note that both referee 2 and 3 are more supportive of publication in EMBO reports and referee 3 considered your "... work on mitophagy in the synapse" as a substantial advance to the field in his/her further feedback. On balance, we would thus like to invite you to revise your manuscript for EMBO reports. Please substantiate the role of Rheb in mitophagy as outlined by referee 1 and 2 and clarify the mitophagy status in the AD background as outlined by referee 3.

Please fully address all referee concerns (as detailed above and in their reports) and take their suggestions 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss 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 providing access to data deposited in public databases is missing (if relevant).
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.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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 (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) 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 your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

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) 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 .

8) 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 .

9) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

10) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. 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.

You are able to opt out of this by letting the editorial office know (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."

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.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

****

Referee #1:

The manuscript by Han & Jeong et al. reports roles for two proteins, Rheb and Snapin, in the maintenance of mitochondrial health at neuronal synapses. The authors propose a new role for Rheb in mediating the mitophagic sequestration of neuronal mitochondria at synapses, and convincingly demonstrate the role of Snapin in mediating retrograde trafficking of mitophagosomes from the synapse. The manuscript is written with adequate clarity, but it would benefit greatly from proofreading by an English-speaking editor.

While the manuscript is well evidenced, it is unclear which aspects of the study represent a novel or conceptual advance. The authors have presented several well-established concepts as though they represent novel hypotheses. For example, the authors state: "Thus, in addition to Aβ-induced mitochondrial damage, our findings allow us to propose that impaired mitochondrial maintenance at AD synapses due to mitophagy failure plays a critical role in mediating synaptic mitochondrial deficit in AD neurons." Not only is this statement an established concept (Reddy & Beal (2008), Trends in molecular medicine), the exact hypothesis has already been studied (Manczak et al. (2018). Human molecular genetics; Fang, et al. (2019), Nature neuroscience).

The findings relating to Rheb and Snapin are reported with a similar degree of overstated novelty. On the topic of Rheb, the authors claim: "we reveal, for the first time, that Rheb-mediated mitophagy is critical for mitochondrial quality control in the axons of healthy and diseased neurons." Rheb expression has been shown to protect against axonal degeneration (Kim, et al. (2012), Molecular Therapy) and promote Nix-Dependent mitophagy (Melser, et al. (2013), Cell metabolism), which has been shown to protect against neurodegeneration (Koentjoro, et al. (2017), Scientific Reports). It is therefore unclear exactly which of the findings presented in this study were revealed for the first time.
Similarly, on the topic of Snapin the authors proposed: "... that defects in dynein-Snapin-mediated retrograde transport exacerbate mitophagic accumulation at AD synapses."

The study convincingly demonstrates the importance of Snapin in the retrograde trafficking of autophagic structures along axons, but the authors have already reported this finding several times before (Tammineni, et al. (2017), Elife; Cai, et al. (2010), Neuron). Autophagic structures are retrieved from axons by regulated retrograde transport (Hollenbeck, P. J. (1993). JCB), and the retrograde axonal trafficking of mitophagosomes has previously been reported (Maday, et al. (2012), JCB). Given that Snapin is required for the retrograde transport of autophagosomes, and that mitophagosomes are autophagosomes, it's unclear how the findings presented in this study represent a conceptual advance over previous reports.

The authors attempt to distinguish their findings from previous studies linking Rheb to mitophagy, by highlighting that mitophagy induction via Rheb was reported to be activated by increased mitochondrial respiration (Melser, et al. (2013), Cell metabolism). In contrast, the present study demonstrates Rheb-dependent mitophagy after mitochondrial depolarization by treating cells with a mitochondrial uncoupler called CCCP. The authors emphasise this difference between the studies, by stating "The current study reveals that Rheb senses $\Delta\psi m$ depolarization". By definition, mitochondrial uncouplers (such as CCCP) uncouple ATP production from the electron transport chain to induce increased respiration. The distinction between these studies claimed by the authors must therefore be substantiated, by demonstrating that Rheb is recruited to depolarized mitochondria in the absence of mitochondrial uncouplers (e.g. Oligomycin/Antimycin A which depolarise mitochondria but also inhibit respiratory activity).

Referee #2:

In the manuscript by Han et al., the authors examine mitophagy in axons and synapses. The results lead the authors to conclude that under normal conditions Rheb GTPase initiates mitophagy in synapses, which then undergo retrograde trafficking. However, using an AD mouse model, they find that although Rheb recruitment to mitochondria is increased, their trafficking is reduced - leading to the accumulation of dysfunctional mitochondria in the synapse. The trafficking defect is related to previous work from the lab on Snapin-mediated transport and here they show that overexpression of Snapin helps reduce this mitochondrial defect and resultant synaptic damage.
On the whole, this is an interesting manuscript and highlights the growing evidence of dysfunctional autophagy/mitophagy and neurodegeneration - in this case AD. Thus, I think this manuscript will of broad interest. However, the results are still largely correlative - disrupting Rheb and Snapin-mediated trafficking will have many cellular consequences outside of mitophagy and it may be these processes that are key for disease pathology. This certainly does not preclude publication in my opinion, but I think a little more work is needed to strengthen the mechanism.
Main Points:

1) I was a little confused over the role Rheb is playing in mitophagy during this instance. Good evidence from the literature suggests that Rheb plays a role in stimulating mitophagy during enhanced mitochondrial function and that this occurs via NIX recruitment to mitochondria (Melser et al., 2013) - a pathway that is independent of PINK1/Parkin. Here the authors are using opposite conditions, i.e. CCCP to completely depolarize mitochondria, which presumably activates PINK1 and Parkin. So, what might the mechanism be here? Is Rheb important for PINK1/Parkin-dependent mitophagy, or is this NIX-dependent mitophagy that the authors are monitoring?
2) Based on the above, are the authors sure it is mitophagy initiation that is controlled by Rheb? Perhaps a higher magnification of the data shown in Fig1A would help, as to my eyes most of the Rheb signal does not look mitochondrial. It looks to form large punctate structures that are adjacent to mitochondria - what are these (endosomes/lysosomes/other autophagic cargo) and could they be relevant?
3) Also, a role for Rheb during mitophagy initiation would be strengthened by showing some high-resolution triple co-localization data with Rheb and LC3 on axonal mitochondria in isolated neurons - the data shown in Fig1E are not sufficient. Do GFP-Rheb (or endogenous) pull downs co-precipitate LC3 upon CCCP treatment?
4) There is extensive evidence for Rheb being late endosomal/lysosomal in localization and it has a very well-established role in mTOR activation. Given the function of lysosomes and mTOR in autophagy, is it possible that the authors are looking at a general defect in autophagy (i.e. is Rheb specific for only mitochondrial autophagy and not other forms)?
5) The tissue staining of LC3 and cytochrome c in various figures would benefit from higher magnification panels. As with the images in isolated neurons, it looks like the blobs of LC3 are adjacent to, and not surrounding mitochondria.
6) In Figure 6 (and related) what are the levels of Snapin overexpression - WT vs L99K?
7) Likewise, what are the levels of Snapin overexpression compared to endogenous in transduced tissue in Figure 7? Is this the data that is in Fig S7? For control, the authors transduce mCherry and for Snapin they transduce mCherry-Snapin - therefore by western blot of Snapin, the size of transduced Snapin should be ~25kDa bigger than endogenous. The bands in S7 are the same size? Perhaps I have misunderstood this? Either way clarification is needed.

Referee #3:

Dr. Qian Cai's team presented a research article entitled 'Mitophagy Regulates Integrity of Mitochondria at Synapses and is Critical for Synaptic Maintenance'. In this paper, they were focused on a very important question: what is the mitophagy status in the synaptic region in both physiological and pathological (Alzheimer's disease) conditions? In combination of primary neuronal culture and mouse brain tissues, with the applications of WB, IHC and dual-channel kymographs, they first time show that the dynein-Snapin-mediated retrograde transport plays an important role in synaptic mitophagy. The data were presented with professionalism and with correct statistics. In summary, a very important study with the findings novel to the broad neuroscience fields, and fits for the EMBO Reports. It is important to address the below questions before qualified for publication.

Major concerns
This reviewer has concerns on the data interpretation in the AD background.
1. Mitophagy status in AD. This study strongly suggests increased autophagy initiation (more LC3II, in line with PMID: 20541250) and increased mitophagosome (Fig. 3 and Fig. 4) in the hAPPmut Tg

mice; but they did not detect changes of mitolysosome in the hAPPmut Tg mice. Thus, it is not appropriate to say 'mitophagy enhancement' (in abstract) or 'mitophagy activation' (Fig. 4 title). It has been very well documented that there is a lysosome defect in AD (PMID: 20541250; PMID: 25991442), and it is very likely that there will be impaired mitolysosome in AD. In addition, mitophagy is impaired in the Tau animals (PMID:30538104). In the Shirley Yang lab, they show that PINK1 signalling rescues AD pathology (PMID: 29077793) in an APP mouse model. The authors' data also suggest a likelihood of impaired autophagy/mitophagy as evidenced by higher p62 (Fig. 3G). At least, it is possible for the authors to evaluate mitophagy (final step) in the primary neurons from the hAPPmut Tg mice using mito-Keima or commercially available Mitophagy dye (Dojindo company).

2. If the coming new data from the authors suggest increased mitophagosome in the hAPPmut Tg mice, then it would be necessary to check mitophagy in the APP/PS1 (with lysosome dysfunction) and or the PS1KO mice (PMID: 20541250). The authors may would like to discuss such different results.

Minor concerns

1. To suggest to change 'release harmful reactive oxygen species (ROS)' to 'release ROS which is harmful at very high concentrations'. It has been well-documented that low (physiological level) ROS is necessary for cells, and even for the longevity of worms (PMID:24813612; PMID: 31801997).

2. Fig. 1B: data of Rheb-SSVm (veh) should be included.

3. Fig. 3A: It would be nice if the authors can give additional justification of using cytochrome C as a mitochondrial marker for this mitophagy evaluation. Since cytochrome C localizes in the intermembrane space, and it will release to cytoplasm in the case of mitochondrial damage. E.g., one can imagine that under the case of UPS (proteasome) to eliminate mitochondrial outmembrane, as well as in the case of damaged mitochondria, there will be cytochrome C releases to cytoplasm, which will increase the colocalization between cytoplasm cytochrome C / cytosome LC3. A suggestion would be cytochrome C oxidase subunit II (COXII), a mitochondrial DNA (mtDNA)-encoded inner membrane protein. The authors may not need to repeat the data, but would be necessary to discuss the potential limitations of using cytochrome C / LC3 as a marker of mitophagy event at the discussion section.

4. Fig. 4E (upper panel WT): for this 'representative image' there was no 'mitophagy' event? Although low, there should have mitophagy event in physiological condition.

5. Additional references should be cited to provide an unbiased overview of the fields: PMID: 20541250; PMID: 25991442; PMID: 31577933; PMID: 30922179; PMID:30538104; PMID:28899755).

Referee #1:

The manuscript by Han & Jeong et al. reports roles for two proteins, Rheb and Snapin, in the maintenance of mitochondrial health at neuronal synapses. The authors propose a new role for Rheb in mediating the mitophagic sequestration of neuronal mitochondria at synapses, and convincingly demonstrate the role of Snapin in mediating retrograde trafficking of mitophagosomes from the synapse. The manuscript is written with adequate clarity, but it would benefit greatly from proofreading by an English-speaking editor.

We appreciate the reviewer's comments. A native English speaker has proofread the revised manuscript.

While the manuscript is well evidenced, it is unclear which aspects of the study represent a novel or conceptual advance. The authors have presented several well-established concepts as though they represent novel hypotheses. For example, the authors state: "Thus, in addition to Aβ-induced mitochondrial damage, our findings allow us to propose that impaired mitochondrial maintenance at AD synapses due to mitophagy failure plays a critical role in mediating synaptic mitochondrial deficit in AD neurons." Not only is this statement an established concept (Reddy & Beal (2008), Trends in molecular medicine), the exact hypothesis has already been studied (Manczak et al. (2018). Human molecular genetics; Fang, et al. (2019), Nature neuroscience).

We appreciate the reviewer's comments, but we respectfully disagree with the reviewer's comment questioning the novel/conceptual advancement that our study provides. The earliest features of the onset of the highly prevalent Alzheimer's disease (AD) have been linked to mitochondrial pathology. Growing evidence has demonstrated that early deficit in synaptic mitochondria is relevant to the development of synaptic pathology in AD. Even though some underlying mechanisms have been proposed, it remains unclear whether mitophagy failure and defects in mitochondrial maintenance are involved. We have acknowledged the established knowledge regarding impaired mitochondrial function in AD, especially at synaptic terminals. We apologize for missing this important publication by Reddy and Beal, 2008 (Trends in Molecular Medicine), which is now cited on **pages 3**, **4 and 14** of the revised manuscript.

As the reviewer correctly noted, mitophagic abnormalities in AD, particularly in the PINK1/Parkin pathway, have been demonstrated in a number of recent studies. Manczak et al., 2018 and Reddy et al., 2018 (Human Molecular Genetics) reported mitophagy defects in AD, as evidenced by reduced levels of PINK1 and TERT. Consistently, our previous work also revealed that PINK1/Parkin-mediated mitophagy is defective in AD patient brains and mouse models (Ye et al., Human Molecular Genetics, 2015). A recent study from Fang et al., 2019 (Nature Neuroscience) further showed that the basal level of mitophagy is reduced in AD brains, whereas enhancement of PINK-1-dependent mitophagy abolishes AD pathology and reverses memory deficits in AD models. Consistently, Yan's group reported that PINK1 overexpression ameliorates cognitive dysfunction in AD mice by promoting the clearance of damaged mitochondria (Du et al., Brain, 2017). Collectively, these studies indicate a critical role of PINK1/Parkin-mediated mitophagy in early AD pathophysiology.

We and others have demonstrated that PINK1/Parkin-mediated mitophagy occurs primarily in the soma of neurons, where degradative lysosomes are highly enriched (Cai et al., Current Biology, 2012; Devireddy et al., Journal of Neuroscience, 2015; Sung et al., Journal of Neuroscience, 2016; Xie et al., Neuron, 2015; Cheng et al., Journal of Cell Biology, 2018; Yap et al., Journal of Cell Biology, 2018). However, critical understanding of the mitochondrial quality control mechanism at nerve terminals remains very limited. In the current study, we uncover a

new mechanism of the quality control of synaptic mitochondria which involves Rheb-dependent mitophagy initiation and dynein-Snapin-mediated retrograde transport. Such a mechanism is essential for the maintenance of mitochondrial homeostasis at synaptic terminals. Furthermore, we hypothesize that increased mitophagy initiation along with defects in retrograde transport results in mitophagic accumulation and mitochondrial stress at AD synapses, thereby exacerbating AD-associated synaptic deficits. More importantly, Snapin-enhanced retrograde transport alleviates mitochondrial defects and mitigates synapse loss in AD mouse brains. Thus, in line with previous understandings of PINK1/Parkin-mediated mitophagy in the soma, our current work not only adds new information on the mechanism of mitochondrial quality control at synaptic terminals, but also provides new insights into the critical role of mitophagy dysfunction in AD-linked synaptic mitochondrial deficit and synaptic pathology.

We have added the above discussion to **pages 14 and15** in the revised manuscript.

The findings relating to Rheb and Snapin are reported with a similar degree of overstated novelty. On the topic of Rheb, the authors claim: "we reveal, for the first time, that Rheb-mediated mitophagy is critical for mitochondrial quality control in the axons of healthy and diseased neurons." Rheb expression has been shown to protect against axonal degeneration (Kim, et al. (2012), Molecular Therapy) and promote Nix-Dependent mitophagy (Melser, et al. (2013), Cell metabolism), which has been shown to protect against neurodegeneration (Koentjoro, et al. (2017), Scientific Reports). It is therefore unclear exactly which of the findings presented in this study were revealed for the first time.

The reviewer raised very insightful points. In the paper by Kim et al., (Molecular Therapy, 2012), Rheb was shown to protect against neurodegeneration and mediate axonal regrowth in dopaminergic neurons. This study concludes that Rheb-associated neuroprotective effect is attributed to activation of the neurotrophic signaling pathway. Thus, this study is different from our current work that is focused on the role of Rheb-mediated mitophagy in mitochondrial maintenance at synaptic terminals.

As the reviewer correctly stated here and later, Mesler et al., (Cell Metabolism, 2013) reveal that Rheb promotes Nix-dependent mitophagy, which is activated particularly upon increased mitochondrial energetics. Moreover, in this study, Rheb-associated mitophagy was only examined in HeLa cells and primary muscle cells. Therefore, the outstanding questions remain: whether mitochondrial damage, but not enhanced mitochondrial function, activates Rheb-associated mitophagy, and whether this mechanism is involved in the control of mitochondrial quality in healthy and diseased neurons. Addressing these new and important questions could also advance our understanding of the mechanisms underlying AD-linked synaptic deterioration, one of the earliest pathologies in stricken neurons.

Koentjoro, et al. (Scientific Reports, 2017) demonstrated that Nix-mediated mitophagy is a PINK1/Parkin-independent pathway. Nix overexpression can improve mitochondrial energy production in PINK1- and Parkin-related PD patient cells. However, this study did not address whether Nix-mediated mitophagy is involved in maintaining mitochondrial integrity at synaptic terminals, whether Rheb plays a role in Nix-mediated mitophagy, or how this mitophagy is regulated in AD neurons.

Different from these above studies, our current work reveals for the first time that: **(1)** Mitochondrial damage activates Rheb-mediated mitophagy in the axons of neurons under both physiological and pathophysiological conditions; **(2)** Rheb-associated mitophagy in axons requires Nix, but is independent of the Parkin-mediated pathway (**new Fig 2D** and **E**, **new Fig**

EV1D-I, and **new Fig EV2A-C and I-J**); **(3)** The role of Rheb is specific to mitophagy, but not other forms of autophagy (**new Fig EV2D-H**); **(4)** Rheb-dependent mitophagy and dynein-Snapin-mediated retrograde transport are crucial for the maintenance of mitochondrial integrity and synaptic homeostasis. **(5)** Mitophagic stress is a prominent feature at AD synapses and caused by increased mitophagy initiation and defective removal of mitophagosomes from AD synapses due to impaired retrograde transport. **(6)** Lysosomal deficiency has no detectable impact on mitophagy in the axons of AD neurons (**new Fig EV4A-I**). **(7)** Mitophagy failure augments synaptic mitochondrial deficit, exacerbating AD-linked synaptic defects. **(8)** Snapin-enhanced retrograde transport alleviates synaptic mitochondrial deficit and ameliorates synapse loss in AD mouse brains. Thus, these findings advance our understanding of the mechanism underlying mitochondrial maintenance through mitophagy at synaptic terminals and also provide new insights into the critical role of mitophagy failure in AD-linked synaptic mitochondrial deficit and synaptic defects.

We have cited these publications and added new data and described the results in the text on **pages 5-7**, **11**, **and 15** of the revised manuscript.

Similarly, on the topic of Snapin the authors proposed: "... that defects in dynein-Snapin-mediated retrograde transport exacerbate mitophagic accumulation at AD synapses."
The study convincingly demonstrates the importance of Snapin in the retrograde trafficking of autophagic structures along axons, but the authors have already reported this finding several times before (Tammineni, et al. (2017), Elife; Cai, et al. (2010), Neuron). Autophagic structures are retrieved from axons by regulated retrograde transport (Hollenbeck, P. J. (1993). JCB), and the retrograde axonal trafficking of mitophagosomes has previously been reported (Maday, et al. (JCB, 2012). Given that Snapin is required for the retrograde transport of autophagosomes, and that mitophagosomes are autophagosomes, it's unclear how the findings presented in this study represent a conceptual advance over previous reports.

We appreciate the reviewer's comments, but respectfully disagree with the reviewer's comments with regard to the novel/conceptual advancement that our study provides. As the reviewer correctly stated, Hollenbeck PJ (JCB, 1993) reported that autophagic organelles undergo regulated transport from distal axons back to the cell body. Maday et al., (JCB, 2012) provided additional information by showing that autophagosomes initiate in distal axons and move in a retrograde direction toward the soma for lysosomal degradation. In our previous studies, we revealed a molecular mechanism regulating autophagy-lysosomal function in neurons through retrograde transport of late endosomes (LEs). We demonstrated that Snapin serves as an adaptor of dynein motors and recruits dynein motors to LEs, enabling long-distance retrograde transport of LEs (Cai et al., Neuron, 2010). In Cheng et al., (JCB 2015), we further showed that nascent autophagosomes in distal axons recruit dynein-Snapin transport machinery to gain retrograde transport motility through fusion with LEs to form amphisomes. Such a mechanism is critical for the delivery of autophagic cargoes from distal axons toward the soma for lysosomal clearance. In a recent study, we found that soluble oligomeric $A\beta_{1-42}$ interacts with dynein motors and this interaction interferes with dynein motor-Snapin coupling, thereby impairing recruitment of dynein motors to autophagic vacuoles (AVs) (Tammineni et al., eLife, 2017). We thus propose that defects in Snapin-dynein-mediated retrograde transport contribute to autophagy dysfunction in AD.

These previous studies, including our own, aim to dissect the general mechanisms of autophagy regulation in healthy and diseased neurons. Our current work, different from these studies, focuses on elucidating the mechanism of mitochondrial quality control through mitophagy at synaptic terminals and its impact on synaptic maintenance. Given early deficit in synaptic

mitochondria at early disease stages of AD (Du et al., PNAS, 2010), advanced understanding of the mitophagy mechanism at synaptic terminals is critical for us to further address whether impaired synaptic mitochondrial maintenance underlies mitochondrial deficit at AD synapses.

Mitophagy is a selective form of autophagy for elimination of dysfunctional mitochondria (Youle and Narendra, Nat Rev Mol Cell Biol 2011; Sheng and Cai Nat Rev Neurosci 2012, Cai and Jeong, Cells, 2020). Upon mitochondrial damage, mitophagy is initiated by recruiting mitophagy-specific machineries to damaged mitochondria followed by sequestration into autophagosomes for lysosomal clearance. While mitophagy is the only known cellular pathway through which entire mitochondria are eliminated within lysosomes, PINK1/Parkin-mediated mitophagy is the most heavily studied and the best-understood mitophagy pathway (Youle and Narendra, Nat Rev Mol Cell Biol 2011; Cai and Jeong, Cells, 2020). We and others uncover that PINK1/Parkin-mediated mitophagy occurs primarily in the soma of neurons (Cai et al., Current Biology, 2012; Devireddy et al., Journal of Neuroscience, 2015; Sung et al., Journal of Neuroscience, 2016; Xie et al., Neuron, 2015; Lin et al., Neuron, 2017). However, the mechanism underlying mitochondrial maintenance at synaptic terminals is still understudied in neurobiology. Our current work sheds light on the molecular details of the regulation of synaptic mitochondrial integrity through Rheb-dependent mitophagy and dynein-Snapin-mediated retrograde transport.

In the revision, we have provided multiple lines of new evidence showing that the Rheb pathway in axons requires Nix, but is independent of Parkin-mediated mitophagy in the soma (**Fig 2D** and **E**, **Fig EV1D-I**, and **Fig EV2A-C and I-J**). Moreover, as Referee #2 suggested, we carefully examined and revealed that the role of Rheb is important particularly for mitophagy, but not other forms of autophagy (**Fig EV2D-H**). Furthermore, we demonstrated that Rheb-associated mitophagosomes are positive for Rab7, a LE/amphisome marker (**Fig EV1F**). This data indicates that newly generated mitophagosomes fuse rapidly with LEs to form amphi-mitophagosomes through which nascent mitophagosomes are loaded with dynein-Snapin transport machinery to enable retrograde transport motility. Therefore, these findings allow us to propose a new mechanism whereby Rheb-dependent mitophagy initiation coordinates with dynein-Snapin-mediated retrograde transport to remove nascent mitophagosomes from distal axons, thus reducing mitochondrial stress at synaptic terminals.

Furthermore, we have revealed that AD-linked synaptic mitophagy stress is characterized by aberrant retention of mito-amphisomes at presynaptic terminals. Mitochondrial and synaptosomal fractions purified from the brains of mutant hAPP mice as well as mitochondrial fractions isolated from cultured AD neurons exhibit increased levels of Rab7 along with Rheb, p62, and LC3-II (**Figs 3** and **4**). These results are consistent with our EM data (**Figs 3** and **4**), suggesting aberrant accumulation of mito-amphisomes at AD synapses. Moreover, AD axons display impaired retrograde transport of mitophagosomes (**Fig 4**). More importantly, Snapin-enhanced retrograde transport reduces mitophagic accumulation and mitochondrial stress in AD axons, mitigating synaptic deficit in AD mouse brains (**Figs 6** and **7**). Collectively, our results reveal that mitophagy stress at AD synapses is caused by increased mitophagy initiation coupled with impaired retrograde transport, which exacerbates mitochondrial pathology and synaptic damage in AD. Therefore, our findings provide new insights into the critical role of mitophagy failure in synaptic mitochondrial deficit and thus advance our understanding of the mechanisms underlying AD-linked synaptic deterioration, one of the earliest pathologies in stricken neurons.

We have added these new data to the revised manuscript and described our new results in the text on **pages 5-8**.

The authors attempt to distinguish their findings from previous studies linking Rheb to mitophagy, by highlighting that mitophagy induction via Rheb was reported to be activated by increased mitochondrial respiration (Melser, et al. (2013), Cell metabolism). In contrast, the present study demonstrates Rheb-dependent mitophagy after mitochondrial depolarization by treating cells with a mitochondrial uncoupler called CCCP. The authors emphasise this difference between the studies, by stating "The current study reveals that Rheb senses Δψm depolarization". By definition, mitochondrial uncouplers (such as CCCP) uncouple ATP production from the electron transport chain to induce increased respiration. The distinction between these studies claimed by the authors must therefore be substantiated, by demonstrating that Rheb is recruited to depolarized mitochondria in the absence of mitochondrial uncouplers (e.g. Oligomycin/Antimycin A which depolarise mitochondria but also inhibit respiratory activity).

The reviewer made an excellent point. Melser et al., 2013 reported that Rheb-mediated mitophagy is induced upon enhanced mitochondrial respiration. As the reviewer correctly noted, CCCP treatment leads to $\Delta\psi_m$ dissipation along with an increase in mitochondrial respiration. Our study proposes that Rheb senses $\Delta\psi_m$ dissipation and mediates the targeting of depolarized mitochondria for autophagy, a new mechanism different from what was found in this previous study. To further strengthen our conclusion, we performed additional experiments to determine whether $\Delta\psi_m$ dissipation activates Rheb-dependent mitophagy. Antimycin A (AA), a mitochondrial electron transport chain complex III inhibitor, has been widely used in the field to study mitochondrial quality control and mitophagy in neurons (Cai et al., Current Biology, 2012; Ashrafi et al., Journal of Cell Biology, 2014; Lin et al., Neuron 2017; Evans and Holzbaur, eLife 2020). More importantly, our previous studies have shown that a low concentration (1 μM) of AA in neurons depolarizes $\Delta\psi_m$, which is coupled with decreased mitochondrial respiration (Cai et al., Current Biology, 2012; Lin et al., Neuron 2017). AA treatment results in a significant increase in Rheb association with depolarized mitochondria within axons, a finding consistent with our observations in CCCP-treated axons (**Fig EV1C and D**, **Fig 1D and E**). Thus, this new result confirms that Rheb is efficiently recruited to mitochondria in response to $\Delta\psi_m$ dissipation. We have added this new data to **Fig EV1C and D** and described it in the text on **page 5** of the revised manuscript.

Referee #2:

In the manuscript by Han et al., the authors examine mitophagy in axons and synapses. The results lead the authors to conclude that under normal conditions Rheb GTPase initiates mitophagy in synapses, which then undergo retrograde trafficking. However, using an AD mouse model, they find that although Rheb recruitment to mitochondria is increased, their trafficking is reduced - leading to the accumulation of dysfunctional mitochondria in the synapse. The trafficking defect is related to previous work from the lab on Snapin-mediated transport and here they show that overexpression of Snapin helps reduce this mitochondrial defect and resultant synaptic damage.
On the whole, this is an interesting manuscript and highlights the growing evidence of dysfunctional autophagy/mitophagy and neurodegeneration - in this case AD. Thus, I think this manuscript will of broad interest. However, the results are still largely correlative - disrupting Rheb and Snapin-mediated trafficking will have many cellular consequences outside of mitophagy and it may be these processes that are key for disease pathology. This certainly does not preclude publication in my opinion, but I think a little more work is needed to strengthen the mechanism.

We are encouraged by the reviewer's positive comments on our study.

Main Points:
1) I was a little confused over the role Rheb is playing in mitophagy during this instance. Good evidence from the literature suggests that Rheb plays a role in stimulating mitophagy during enhanced mitochondrial function and that this occurs via NIX recruitment to mitochondria (Melser et al., 2013) - a pathway that is independent of PINK1/Parkin. Here the authors are using opposite conditions, i.e. CCCP to completely depolarize mitochondria, which presumably activates PINK1 and Parkin. So, what might the mechanism be here? Is Rheb important for PINK1/Parkin-dependent mitophagy, or is this NIX-dependent mitophagy that the authors are monitoring?

The reviewer raised excellent points. As the reviewer correctly noted, Melser et al., 2013 reported that increased mitochondrial respiration induces Rheb-mediated mitophagy, a Nix-dependent but Parkin-independent pathway. Our current study proposes a new mechanism by which Rheb-mediated mitophagy is activated in the axons of neurons upon mitochondrial damage. We demonstrated activation of this mitophagy not only in axons treated with CCCP, but also in the axons of Alzheimer's disease (AD)-related mutant hAPP neurons in the absence of $\Delta\psi_m$ dissipation reagents as well as in AD mouse brains. As Referee #1 suggested, we have provided new evidence showing that this pathway can be induced within axons treated with a low concentration of Antimycin A (AA), a mitochondrial electron transport chain complex III inhibitor. Our previous studies have shown that AA treatment depolarizes $\Delta\psi_m$, which is coupled with decreased mitochondrial respiration (Cai et al., Current Biology, 2012; Lin et al., Neuron 2017). After treatment with AA, we detected a significant increase in Rheb localization to depolarized mitochondria within axons, a finding consistent with our observations in CCCP-treated axons (**Fig EV1C and D**, **Fig 1D and E**). Thus, our results consistently suggest that Rheb senses $\Delta\psi_m$ depolarization to initiate mitophagy in axons. We have added this new data to **Fig EV1C and D** and described it in the text on **page 5** of the revised manuscript.

To address the reviewer's comments of whether Rheb plays a role in Parkin- or Nix-dependent mitophagy, we performed multiple lines of new experiments. We first examined whether Rheb is important for Parkin-mediated mitophagy in neurons. We and others have shown that Parkin-mediated mitophagy primarily occurs in the soma of neurons, where degradative lysosomes are

highly enriched (Cai et al., 2012; Devireddy et al., 2015; Sung et al., 2016; Xie et al., 2015; Cheng et al., 2018; Yap et al., 2018). CCCP treatment robustly activates Parkin-mediated mitophagy in the soma, as reflected by Parkin translocation onto depolarized mitochondria. However, Rheb RNAi does not lead to a detectable change in the percentage of neurons showing Parkin association with mitochondria, suggesting that Rheb does not play a role in Parkin-dependent mitophagy in the soma (**Fig EV2I and J**). Moreover, axons expressing Parkin shRNA does not display defects in Rheb localization to depolarized mitochondria (data not shown) and mitophagosome biogenesis after CCCP treatment (**Fig 2D and E**). Thus, our new data suggest that Parkin is not involved in Rheb-mediated mitophagy in axons.

We next examined whether Rheb-mediated mitophagy requires Nix, an outer mitochondrial membrane protein (Youle and Narendra, 2011; Cai and Jeong, 2020). Nix RNAi results in a marked reduction of Rheb localization to mitochondria in response to $\Delta\psi_m$ dissipation (**Fig EV1G and H**). Moreover, Rheb-mediated targeting of mitochondria for autophagy is significantly impaired in axons expressing Nix shRNA, as evidenced by decreased numbers of Rheb-AVs and mitophagosomes (**Fig EV1I and J** and **Fig 2D and E**). Furthermore, in transfected HeLa cells, Rheb-LC3-II complex was detected after treatment with CCCP, but not DMSO control. Nix is associated with Rheb-LC3-II complex (**Fig EV2B and C**). Consistently, Rheb RNAi significantly reduces the number of mitophagosomes within axons (**Fig 2D and E**).

Taken together, we propose that Rheb-associated mitophagy requires Nix and mediates the targeting of damaged mitochondria for autophagy in axons, which parallels Parkin-dependent mitophagy in the soma. Our results are consistent with previous work showing that Nix-mediated mitophagy is a PINK1/Parkin-independent pathway (Koentjoro, et al. 2017). We have added these new data to **Fig 2D and E**, **Fig EV1G-J**, and **Fig EV2B and C** and described the results in the text on **pages 5-8** of the revised manuscript.

2) Based on the above, are the authors sure it is mitophagy initiation that is controlled by Rheb? Perhaps a higher magnification of the data shown in Fig1A would help, as to my eyes most of the Rheb signal does not look mitochondrial. It looks to form large punctate structures that are adjacent to mitochondria - what are these (endosomes/lysosomes/other autophagic cargo) and could they be relevant?

The reviewer raised great points. To address the reviewer's concerns, in new **Figure 1B**, we provided time-lapse images showing that Rheb co-localizes and co-migrates with mitochondria along axons treated with CCCP. Interestingly, Rheb-tagged mitochondria exhibit high motility and move exclusively in a retrograde direction (**Fig 1A and B**). This is consistent with our data that Rheb is associated with LC3-marked autophagic vacuoles (AVs) undergoing predominant retrograde movement in axons (**Fig 1F and 1H**). Moreover, we have provided additional images showing Rheb localization to mitochondria upon mitochondrial damage in WT and AD neurons (**Fig 1E and Fig 4A**). As the reviewer suggested, our new triple-channel image data further demonstrated that Rheb-associated autophagosomes contain engulfed mitochondria within axons, suggesting that they are mitophagosomes in nature (**Fig EV1E**). Our results are consistent with previous studies showing mitochondria colocalized and cotransporting with autophagosomes in a retrograde direction along axons (Maday et al., 2012; Wong and Holzbaur, 2014). This supports the possibility that mitophagy plays a role in regulating proper turnover of axonal mitochondria.

We previously reported that nascent AVs recruit dynein-Snapin, a motor-adaptor complex, to gain retrograde transport motility through fusion with late endosomes (LEs) to form amphisomes. Such a mechanism facilitates autophagic clearance within lysosomes in the soma

(Cheng et al., J Cell Biol, 2015). In the revision, we have further demonstrated that Rheb-associated mitophagosomes are mostly positive for Rab7, an LE/amphisome marker (**Fig EV1F**). This data suggests that newly generated mitophagosomes fuse rapidly with LEs to form amphi-mitophagosomes, through which nascent mitophagosomes are loaded with dynein-Snapin transport machinery to enable retrograde transport motility. Consistently, mitochondrial and synaptosomal fractions purified from the brains of mutant hAPP mice and *snapin*-mutant mice, as well as mitochondrial fractions isolated from cultured AD neurons, displayed increased levels of Rab7 along with Rheb, p62, and LC3-II (**Figures 3**-**5**). These results are also consistent with our EM data (**Figs 3**-**5**), suggesting that mito-amphisomes are aberrantly accumulated at synaptic terminals. Therefore, these findings allow us to propose that Rheb-dependent mitophagy initiation coordinates with dynein-Snapin-mediated retrograde transport to remove damaged mitochondria from synaptic terminals. Defects in such a mechanism result in mitophagic stress at AD synapses.

These new data have been added to **Fig 1B and E**, **Fig 4A**, and **Fig EV1E and F** and described in the text on **pages 5-6** of the revised manuscript.

3) Also, a role for Rheb during mitophagy initiation would be strengthened by showing some high-resolution triple co-localization data with Rheb and LC3 on axonal mitochondria in isolated neurons - the data shown in Fig1E are not sufficient. Do GFP-Rheb (or endogenous) pull downs co-precipitate LC3 upon CCCP treatment?

The reviewer suggested great experiments. Our new triple-channel image data have shown that Rheb-associated AVs in axons contain engulfed mitochondria, suggesting that they are mitophagosomes in nature (**Fig EV1E**).

As the reviewer suggested, we performed a series of co-immunoprecipitation assays and have demonstrated that Rheb forms a complex with LC3-II in cultured cortical neurons in the presence of CCCP (**Fig EV2A**). In transfected HeLa cells, the Rheb-LC3-II complex formation was detected upon treatment with CCCP, but not DMSO control. Moreover, Nix is associated with the Rheb-LC3-II complex (**Fig EV2B and C**). Our data suggest that upon $\Delta\psi_m$ dissipation, Rheb promotes mitophagy by forming a complex with Nix and LC3-II. Given that Parkin is not present in HeLa cells, this result indicates that such a mechanism is independent of the Parkin pathway.

We have added the new data to **Fig EV1E** and **Fig EV2A-C** and described the results in the text on **pages 5-7** of the revised manuscript.

4) There is extensive evidence for Rheb being late endosomal/lysosomal in localization and it has a very well-established role in mTOR activation. Given the function of lysosomes and mTOR in autophagy, is it possible that the authors are looking at a general defect in autophagy (i.e. is Rheb specific for only mitochondrial autophagy and not other forms)?

The reviewer made excellent comments. As the reviewer correctly noted, Rheb is well established as an activator of mTOR. However, the role of Rheb in mitophagy has been previously shown to be independent of that in Rheb-mediated mTOR activation (Melser et al., 2013). To further address this issue, we carefully examined whether Rheb is specific to mitophagy, but not other forms of autophagy. We and others have shown that trehalose induces non-selective autophagy in neurons (Kruger et al., 2012; Feng et al., 2017). Consistently, trehalose treatment markedly increases the number of AVs within axons (**Fig EV2D and E**). Importantly, Rheb RNAi has no detectable impact on trehalose-induced autophagy in axons, as

evidenced by the unaltered numbers of AVs and mitophagosomes (**Fig EV2D-G**). Moreover, Rheb is not associated with AVs and mitochondria in axons upon autophagy induction by trehalose (**Fig EV2H**). Thus, these results suggest that Rheb plays a critical role in mitophagy, but not in non-selective autophagy. We have added the new data to **Fig EV2D-H** and described this result in the text on **page 7** in the revised manuscript.

5) The tissue staining of LC3 and cytochrome c in various figures would benefit from higher magnification panels. As with the images in isolated neurons, it looks like the blobs of LC3 are adjacent to, and not surrounding mitochondria.

The reviewer raised a great point. We have shown more images of LC3 and cytochrome *c* staining with high magnification (**Fig 3C**). Also, we did more staining by using alternative antibodies against p62, an autophagy marker, and HSP60, a mitochondrial matrix protein. Consistently, we observed aberrant accumulation of mitophagosomes co-labeled by p62 and HSP60 in hippocampal mossy fibers and surrounding amyloid plaques in mutant hAPP mouse brains (**Fig 3C**, **Fig EV3A and B**, and **Fig EV5E and F**). We have added these new data to the revised manuscript and described the results in the text on **pages 9 and 13**.

6) In Figure 6 (and related) what are the levels of Snapin overexpression - WT vs L99K?

Low transfection efficiency of cultured primary neurons does not allow us to measure Snapin expression by western blot analysis. Instead, we demonstrated that the expression levels of HA-Snapin and HA-Snapin-L99K are similar in transfected HEK293 cells (**Fig EV5B**). We have added this data and described them in the text on **pages 13 and 35** of the revised manuscript.

7) Likewise, what are the levels of Snapin overexpression compared to endogenous in transduced tissue in Figure 7? Is this the data that is in Fig S7? For control, the authors transduce mCherry and for Snapin they transduce mCherry-Snapin - therefore by western blot of Snapin, the size of transduced Snapin should be ~25kDa bigger than endogenous. The bands in S7 are the same size? Perhaps I have misunderstood this? Either way clarification is needed.

Given that the size of Snapin (136 aa) is smaller than mCherry, we used the AAV2/9-mCherry-Snapin construct, which contains an IRES segment between mouse Snapin and mCherry sequences, resulting in separate expressions of mouse Snapin and mCherry (Xie et al., Neuron 2015; Ye et al., J Neurosci 2017). Thus, the size of the transduced Snapin is the same as endogenous Snapin in mouse brains after AAV injection. We have made this clarification in the text on **page 31** of the revised manuscript.

Referee #3:

Dr. Qian Cai's team presented a research article entitled 'Mitophagy Regulates Integrity of Mitochondria at Synapses and is Critical for Synaptic Maintenance'. In this paper, they were focused on a very important question: what is the mitophagy status in the synaptic region in both physiological and pathological (Alzheimer's disease) conditions? In combination of primary neuronal culture and mouse brain tissues, with the applications of WB, IHC and dual-channel kymographs, they first time show that the dynein-Snapin-mediated retrograde transport plays an important role in synaptic mitophagy. The data were presented with professionalism and with correct statistics. In summary, a very important study with the findings novel to the broad neuroscience fields, and fits for the EMBO Reports. It is important to address the below questions before qualified for publication.

We appreciate the reviewers' positive comments on our work.

Major concerns

This reviewer has concerns on the data interpretation in the AD background.
1. Mitophagy status in AD. This study strongly suggests increased autophagy initiation (more LC3II, in line with PMID: 20541250) and increased mitophagosome (Fig. 3 and Fig. 4) in the hAPPmut Tg mice; but they did not detect changes of mitolysosome in the hAPPmut Tg mice. Thus, it is not appropriate to say 'mitophagy enhancement' (in abstract) or 'mitophagy activation' (Fig. 4 title). It has been very well documented that there is a lysosome defect in AD (PMID: 20541250; PMID: 25991442), and it is very likely that there will be impaired mitolysosome in AD. In addition, mitophagy is impaired in the Tau animals (PMID:30538104). In the Shirley Yang lab, they show that PINK1 signalling rescues AD pathology (PMID: 29077793) in an APP mouse model. The authors' data also suggest a likelihood of impaired autophagy/mitophagy as evidenced by higher p62 (Fig. 3G). At least, it is possible for the authors to evaluate mitophagy (final step) in the primary neurons from the hAPPmut Tg mice using mito-Keima or commercially available Mitophagy dye (Dojindo company).

The reviewer raised really great points with regard to the potential impact of lysosomal defects on mitophagic abnormalities at AD synapses since mitophagy is a lysosome-dependent pathway. It is well established that proteolytically active lysosomes with full activity of lysosomal hydrolases are highly enriched in the soma of neurons (Cai et al., 2010; Cai et al., 2012; Xie et al., 2015; Gowrishankar et al., 2015; Maday and Holzbaur, 2016; Tammineni et al., 2017; Cheng et al., 2018; Yap et al., 2018; Lee et al., 2019). Consistent with predominantly somatic localization of mature lysosomes in neurons, previous studies have demonstrated that acidic mitochondria, visualized by mt-Keima, can be detected in the soma, but not in axons (Bingol et al., 2014; Puri et al., 2019). We and others have shown that impaired lysosomal proteolysis leads to lysosomal accumulation of undigested substrates, including autophagy cargoes, in the neuronal soma (Cai et al., 2010; Xie et al., 2015; Maday and Holzbaur, 2016; Tammineni et al., 2017; Cheng et al., 2018; Lee et al., 2019).

In the revision, we utilized mt-Keima to examine the distribution of mitochondria within acidic lysosomal organelles upon mitochondrial damage and lysosomal inhibition. mt-Keima is a ratiometric pH-sensitive fluorescent probe that targets to the mitochondrial matrix. A short wavelength (440 nm) is predominant for excitation in a neutral environment (neutral mito), whereas a long wavelength (586 nm) is predominant in an acidic environment (acidic mito) (Katayama et al., 2011). We found that acidic mitochondria are exclusively located in the soma of neurons (**Fig EV4A and B**). The number of acidic mitochondria is elevated following CCCP

treatment, and we observed additional increase in neurons when treated with CCCP and lysosomal inhibitors (LIs). However, acidic mitochondria cannot be detected in axons under any of these conditions (**Fig EV4A**). Furthermore, we have confirmed that these acidic mitochondria are retained within LAMP1-labeled lysosomes in the soma of neurons. The same treatments lead to a significant increase in the number of mitolysosomes (**Fig EV4C and D**). Consistent with studies from other groups (Bingol et al., 2014; Puri et al., 2019), these findings support the notion that mitophagic clearance mainly occurs in the soma of neurons. Therefore, efficient retrograde transport plays a critical role in removing newly generated mitophagosomes from distal axons for their clearance within somatic lysosomes, thereby reducing mitophagy stress at synaptic terminals.

Given that lysosomal deficiency has been implicated as one of the main cell defects in AD (Nixon, 2013; Menzies et al., 2015), we next examined whether mitolysosomes or acidic mitochondria are accumulated in AD neurons. Compared to WT controls, mitolysosomes are retained in the soma of mutant hAPP neurons, and such retention is augmented upon $\Delta\psi_m$ dissipation (**Fig EV4E and F**). This is consistent with enhanced accumulation of acidic mitochondrial in the soma of AD neurons (**Fig EV4G and H**). However, we did not detect acidic mitochondria in AD axons with or without CCCP treatment (**Fig EV4I**). We and others have demonstrated that Parkin-mediated mitophagy primarily occurs in the soma of neurons (Cai et al., 2012; Ye et al., 2015; Devireddy et al., 2015; Sung et al., 2016; Lin et al., 2017). We further showed that Parkin-mediated mitophagy is robustly induced in mutant hAPP neurons (Ye et al., 2015). Moreover, our previous work revealed lysosomal protease deficiency and impaired lysosomal proteolysis of autophagic substrates in this mutant hAPP Tg (J20) mouse model (Tammineni et al., 2017). Thus, mitolysosomal retention in the soma of AD neurons could be attributed to Parkin-mediated mitophagy induction and defects in lysosomal proteolysis. Therefore, our results indicate that lysosomal dysfunction has no direct impact on mitophagy regulation at synaptic terminals, further supporting the view that mitophagy stress at AD synapses is caused by increased mitophagy initiation and defective retrograde transport of mitophagosomes.

Given that mitophagic clearance within lysosomes occurs primarily in the soma of neurons, increased levels of p62 in both synapse-enriched synaptosomal preparations and mitochondrial fractions purified from mutant hAPP Tg mouse brains are most likely due to mitophagic retention at synaptic terminals (**Fig 3F-I**). *snapin*-deficient mice recapitulate such defects showing elevated p62 levels in synaptosomal and mitochondrial preparations (**Fig 5C-F**). This is further supported by our new data from light imaging studies that mitophagosomes co-labeled by HSP60 and p62 accumulate within the hippocampal mossy fibers and dystrophic presynaptic terminals surrounding amyloid plaques in mutant hAPP Tg mouse brains (**Fig 3C** and **Fig EV3A and B**). Moreover, Snapin-enhanced retrograde transport promotes axonal removal of these mitophagosomes and thus reduces mitophagic stress in distal axons, as evidenced by decreased number of mitophagosomes co-labeled by HSP60 and p62 (**Fig EV5E and F**). We have added these new data to **Fig 3C**, **Fig EV3A and B**, **Fig EV4A-I**, and **Fig EV5E and F** and described these results in the text on **pages 9**, **11-13**, **and 16-17** of the revised manuscript.

As the reviewer correctly noted, mitophagic abnormalities in AD, particularly in the PINK1/Parkin pathway, have been reported in a number of recent studies (Ye et al., 2015; Du et al., 2017; Manczak et al., 2018; Reddy et al., 2018; Cummins et al., 2019). For instance, our studies revealed that Parkin-mediated mitophagy is induced at early disease stages in AD brains, cytosolic depletion of Parkin over disease progression results in defective mitophagy and aberrant accumulation of damaged mitochondria in AD neurons (Ye et al., 2015). Defects in Parkin-mediated mitophagy were also reported in neuroblastoma cells and *Caenorhabditis*

elegans nervous system under tauopathy conditions (Cummins et al., 2019). Manczak et al., 2018 and Reddy et al., 2018 reported reduced levels of PINK1 and TERT in AD brains. More importantly, Yan's group elegantly demonstrated that PINK1 overexpression ameliorates cognitive dysfunction in AD mice by promoting the clearance of damaged mitochondria (Du et al., 2017). A recent study from Fang et al., 2019 further demonstrated that the basal level of mitophagy is also reduced in AD brains. This study is consistent with the findings from Yan's group showing that enhancement of PINK-1-dependent mitophagy abolishes AD pathology and reverses memory deficits in AD models. Collectively, these data indicate a critical role of PINK1/Parkin-mediated mitophagy in AD pathophysiology.

Given that Parkin-mediated mitophagy primarily occurs in the soma of neurons (Cai et al., 2012; Ye et al., 2015; Devireddy et al., 2015; Sung et al., 2016; Lin et al., 2017), this raises the possibility that alternative mechanism(s) must function to efficiently remove constantly damaged mitochondria from axonal terminals to maintain mitochondrial homeostasis under disease-associated chronic stress conditions. In the current study, we uncover a new mechanism of the quality control of synaptic mitochondria which involves Rheb-dependent mitophagy and dynein-Snapin-mediated retrograde transport. Such a mechanism is essential for the maintenance of mitochondrial homeostasis at synaptic terminals. Furthermore, we hypothesize that increased mitophagy initiation along with defects in retrograde transport results in mitophagic accumulation and mitochondrial stress at AD synapses, thereby exacerbating AD-linked synaptic mitochondrial deficit and synaptic damage. Importantly, Snapin-enhanced retrograde transport alleviates mitophagy dysfunction and mitigates synapse loss in AD mouse brains. In the revision, we have provided multiple lines of new evidence showing that the Rheb pathway in axons requires Nix, but is independent of Parkin-mediated mitophagy in the soma (**Fig 2D and E**, **Fig EV1D-I**, and **Fig EV2A-C and I-J**). Moreover, as Referee #2 suggested, we carefully examined and revealed that the role of Rheb is specific to mitophagy, but not other forms of autophagy (**Fig EV2D-H**). We have added these new data to the text on **pages 6-8** and **15** in the revised manuscript. As the reviewer suggested, we modified the title of Fig 4 as "increased mitophagy initiation coupled with defective retrograde transport in the axons of mutant hAPP Tg neurons" on **pages 10 and 21**.

2. If the coming new data from the authors suggest increased mitophagosome in the hAPPmut Tg mice, then it would be necessary to check mitophagy in the APP/PS1 (with lysosome dysfunction) and or the PS1KO mice (PMID: 20541250). The authors may would like to discuss such different results.

The reviewer raised very insightful points. As the reviewer correctly stated, lysosomal defects have been indicated in APP/PS1 Tg and PS1KO mice and cells (Lee et al., 2010; Zhang et al., 2012; Neely Kayala et al., 2012; Hung and Livesey, 2018). Whether lysosomal deficiency impacts mitophagy in AD neurons is an important question to address. We currently do not have the APP/PS1 Tg mice and PS1 KO mice in the lab. We were unable to arrange the shipment of the APP/PS1 Tg mice due to new regulations of the animal care at Rutgers in response to the COVID-19 circumstances. Alternatively, we addressed this important question by transfecting mutant hAPP neurons with Familial AD (FAD)-associated mutations PS1L286E or PS1L286V, respectively (Kulic et al., PNAS, 2000). Compared to mutant hAPP neurons, we observed enhanced mitolysosomal retention in the soma of mutant hAPP neurons co-expressing PS1L286E or PS1L286V (**Fig EV4E and F**). Such a defect is augmented upon $\Delta\psi_m$ dissipation. While this observation is consistent with aberrant accumulation of acidic mitochondria in the soma, we did not detect acidic mitochondria within axons of AD neurons expressing mutant PS1 in the presence or absence of CCCP (**Fig EV4G-I**). Taken together, we propose that mitophagy stress at AD synapses is attributed to elevated Rheb-mediated mitophagy initiation and

defective retrograde transport of mitophagosomes, but not abnormal accumulation of mitolysosomes. We have added these new data to **Fig EV4E-I** and described the results in the text on **pages 10-11** of the revised manuscript.

Minor concerns

1. To suggest to change 'release harmful reactive oxygen species (ROS)' to 'release ROS which is harmful at very high concentrations'. It has been well-documented that low (physiological level) ROS is necessary for cells, and even for the longevity of worms (PMID:24813612; PMID: 31801997).

We have made the change accordingly on **Page 3**.

2. Fig. 1B: data of Rheb-SSVm (veh) should be included.

We have included the data of Rheb-SSVM (Veh) in new **Fig 1D and 1G (**old **Fig 1B and 1F)**.

3. Fig. 3A: It would be nice if the authors can give additional justification of using cytochrome C as a mitochondrial marker for this mitophagy evaluation. Since cytochrome C localizes in the intermembrane space, and it will release to cytoplasm in the case of mitochondrial damage. E.g., one can imagine that under the case of UPS (proteasome) to eliminate mitochondrial outmembrane, as well as in the case of damaged mitochondria, there will be cytochrome C releases to cytoplasm, which will increase the colocalization between cytoplasm cytochrome C / cytosome LC3. A suggestion would be cytochrome C oxidase subunit II (COXII), a mitochondrial DNA (mtDNA)-encoded inner membrane protein. The authors may not need to repeat the data, but would be necessary to discuss the potential limitations of using cytochrome C / LC3 as a marker of mitophagy event at the discussion section.

The reviewer made an excellent point. As the reviewer correctly stated about the possibility of cytochrome *c* (Cyto c) release from damaged mitochondria into the cytoplasm, we examined mitophagic accumulation by using alternative antibodies against p62 and HSP60, a mitochondrial matrix protein. The new immunostaining data consistently show that mitophagosomes co-labeled by HSP60 and p62 accumulate within axons and at dystrophic presynaptic terminals in hippocampal mossy fiber regions of AD mouse brains (**Fig 3C**, **Fig EV3A and B**, and **Fig EV5E and F**). These imaging data are also consistent with the results from biochemical studies and EM analysis as well as the data from cultured neurons (**Fig 3, Fig 4,** and **Fig EV3**), collectively suggesting mitophagic accumulation at AD synapses. We have added the new data to the revised manuscript and described them in the text on **pages 9 and 13**.

4. Fig. 4E (upper panel WT): for this 'representative image' there was no 'mitophagy' event? Although low, there should have mitophagy event in physiological condition.

As the reviewer correctly noted, we have replaced that kymograph image in new **Fig 4C** (old **Fig 4E)**.

5. Additional references should be cited to provide an unbiased overview of the fields: PMID: 20541250; PMID: 25991442; PMID: 31577933; PMID: 30922179; PMID:30538104; PMID:28899755).

We have cited these publications on **pages 3**, **11**, **and 14-15** in the revised manuscript.

1st Revision - Editorial Decision 29th May 2020

Dear Dr. CAI

Thank you for the submission of your revised manuscript to EMBO reports. Your manuscript was evaluated by former referee 2 and 3 and we have now received their reports (copied below).

As you will see, both referees are very positive about the study and support publication without further revisions.

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 note that EMBO Reports will change from the current numbered reference style to the Harvard style as of July 1st (date of publication). It is not mandatory to change the reference style now but in case it does not cause too much troubles, you might want to adjust the references. The respective EndNote file is available here
https://endnote.com/style_download/embo-reports/

2) We noted that you refer to "Data not shown" in several instances (pages 6, 7, 9, 11). Please note that our editorial policies strongly recommend showing all relevant data in the manuscript. Therefore, please either include the referenced data (or remove the respective statements).

3) Please provide scale bars for the magnifications shown in Fig 3, Fig 5, and Fig EV3 and define their size in the legend.

4) Manuscript organization:
Please reorganize the individual manuscript sections that follow the Discussion paragraph in this order:
Discussion
Materials and Methods
Acknowledgements
Author contributions
Conflict of Interest
References
Figure legends
Expanded View figure legends

5) Please also note that a "Data availability" section at the end of Materials and Methods is mandatory. You can state that you have not generated data that require deposition in a public database in this section.

6) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

7) 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 550x200-400 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.

Yours sincerely,

Martina Rembold, PhD
Editor
EMBO reports

****

Referee #2:

I'm happy that the authors have addressed my concerns.

Referee #3:

The authors have done an excellent job in addressing the questions raised by this reviewer and the other reviewers, as evidenced by all the new data clearly and precisely presented.

No additional questions available.

May 31st, 2020

Editor, EMBO Reports

Dear Dr. Rembold,

Thank you for your decision letter with regard to our manuscript (EMBOR-2019-49801V2) entitled "**Mitophagy Regulates Integrity of Mitochondria at Synapses and is Critical for Synaptic Maintenance**". Now, we submit our revision for your consideration for publication in ***EMBO Reports***. We also provide our point-by-point response to your suggestions as highlighted below:

1. We have changed the references to the new style in the revised manuscript.

2. We have removed the respective sentences with "data not shown" in the revised manuscript.

3. We have provided scale bars for the magnifications shown in Fig 3, Fig 5, Fig7, and Fig EV3 and define their sizes in the legend, respectively.

4. We have reorganized the manuscript in the order as you suggested.

5. We have included a statement in the "Data availability" section.

6. We have addressed all the comments raised by the data editor and revised the manuscript with tracked changes.

7. We provided a short summary, 3 bullet points, and a synopsis image.

We hope that our improved manuscript is now acceptable for publication in ***EMBO Reports***.

Thank you for your consideration of our revision,

Qian Cai, M.D., Ph.D.
Associate Professor
Department of Cell Biology and Neuroscience
Rutgers, The State University of New Jersey USA
Phone: 848-445-1633
Email: cai@biology.rutgers.edu
Webpage: http://cbn.rutgers.edu/cb-profile/userprofile/qc29

2nd Revision - Editorial Decision 5th Jun 2020

Dr. Qian CAI
Rutgers, The State University of New Jersey
Cell Biology and Neuroscience
604 Allison Road, Nelson Labs, Room B231
Piscataway 08854
United States

Dear Dr. CAI,

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,

Martina Rembold, PhD
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-2019-49801V3 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: Qian Cai
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49801V1

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 $\chi 2$ 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?	We have followed the standard established in the field to determine the sample size for analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The animal studies are from at least three pairs of animals from each group.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples and animals are all included for analysis unless the experiments are failed due to technical issues.
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, treatment and analysis have been performed blindly to condition during the entire process of the study.
For animal studies, include a statement about randomization even if no randomization was used.	The animal studies are randomized.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, treatment and analysis have been performed blindly to condition during the entire process of the study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The animal studies have been performed blindly to condition.
5. For every figure, are statistical tests justified as appropriate?	Yes, we have followed the standard established in the field.
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 information has been provided.
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 information has been provided.

* 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.	The information has been provided.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	The information has been provided.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	N/A
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).	N/A
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.	N/A

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.	No.