THE EMBO JOURNAL

ATAD3B is a mitophagy receptor mediating clearance of oxidative stress-induced damaged mitochondrial DNA

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DOI: 10.15252/embj.2020106283

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Review Timeline:

Submission Date:	18th Jul 20
Editorial Decision:	13th Aug 20
Revision Received:	25th Nov 20
Editorial Decision:	17th Dec 20
Revision Received:	22nd Jan 21
Accepted:	1st Feb 21

Editor: Elisabetta Argenzio

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 13th Aug 2020

Thank you for submitting your manuscript entitled "ATAD3B Mediates the Clearance of Damaged mtDNA by Acting as a Mitophagy Receptor" (EMBOJ-2020-106283) to The EMBO Journal. Your study has been sent to three referees for evaluation and we have now received reports from them, which are enclosed below for your information.

As you can see, while the referees find your work potentially interesting, they also raise major points that need to be addressed before they can support publication in The EMBO Journal. We agree with the referees that these are important issues and addressing them would be essential to pursue publication of this study in The EMBO Journal. Strong support from the referees would also be needed for publication here. Given the overall interest of your study, I would like to invite you to submit a revised version of the manuscript according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content published elsewhere.

Before submitting your revised manuscript, deposit any primary datasets (and computer code, where appropriate) produced in this study in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password, in case the datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

Feel free to contact me if you have any questions about the submission of the revised manuscript to The EMBO Journal. I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Referee #1:

Manuscript summary
Shu et al. report the identification of a novel mitophagy receptor, ATAD3B, that mediates the clearance of mitochondria upon oxidative stressed-induced mtDNA damage. Firstly, the authors show that oxidative stressors, 3-NPA and H2O2 respectively, lead to irreparable mtDNA damage and provide evidence that the autophagy machinery is necessary to clear mtDNA damage by use of stable ATG5 knockdown cell lines. In order to assess possible regulators/initiators of mtDNA damage induced mitophagy, stable knockdown cell lines, targeting different mtDNA-binding or

associated proteins, were compared in their efficiency to perform mitophagy using mito-Keima assays as readout. They identified ATAD3 as necessary factor for mitophagy events and state that isoform B promotes mitophagy in a PINK1-independent manner by performing mitophagy assays in PINK1 KO background. Further, they showed a direct interaction between ATAD3B and LC3 via its C-terminal LIR motif which is lacking in the 3A isoform. Next, Shu and colleagues claim that mtDNA damage induces a change of localization of ATAD3B from intermembrane space to outer mitochondrial membrane (OMM) by performing colocalization immunofluorescence stainings with OMM protein TOM20. Further, they analyzed the localization by gradual digestion of isolated mitochondria with proteinase K and analysis of the protein content via immunoblot. Their model implies that oxidative stress leading to DNA damage results in translocation to the outer membrane where the LIR region is getting accessible for LC3 binding and recruitment of the autophagy machinery. Furthermore, they state that ATAD3A and B undergo less oligomer formation upon oxidative stress. Finally, they could show that the pathogenic point mutation 3243 A>G, which causes MELAS, induces oxidative stress and that MELAS patient-derived fibroblasts hardly express ATAD3B. Reconstitution experiments with ATAD3B-Flag but not ATAD3A-Flag resulted in clearance of mutated mtDNA showing the protective role of ATAD3B not only under chemically induced but also pathophysiological oxidative stress.

Major comments

1) The authors should explain how the frequency of mtDNA damage, which is shown in Figure 1 B and S1 B is actually calculated from the mtDNA amplification data. Further, in material and methods section it is not clear what is meant by "1/2 control was necessary for loading error".

2) It is not clear how the fluorescence intensity from immunofluorescence stainings was quantified since it is neither explained in the materials and methods section nor in figure legends. This information should be included: Was the fluorescence intensity of the whole image quantified or only prominent dots or just the enlarged sections? Also, it is not always mentioned in the figure legend how many replicates were used for the quantification e.g. Figure S3 C+E

3) For some cell lines used in this study, no validation is shown. Immunoblot analysis for prove of proper stable knockdown should be added for following cell lines:

a. Figure 2A: shLONP1, shSSBP1, shPEO1, shPOLG2, shPOLRMT, shTAM, shATAD3, shATG5 HeLa

b. Figure 3A: PINK1 KO shATAD3B HeLa

4) Figure 1 G hardly shows colocalization events between GFP-LC3 and DNA/TOM20. Thus, one cannot state that these treatments induce specifically mitophagy, since the increase of GFP-LC3 could be also a general increase in autophagy. This experiment should be repeated with a quantification of colocalizing punctae.

5) Figure 3: Figure 3H and J are not describing the antibody used for the first staining panel. The figure legend says anti-Flag antibody but since PINK1 KO cells without Flag-tagged reconstitution were used, this seems not plausible. Further, in the DMSO treated cells no shATAD3B PINK1 KO HeLa cells are depicted, why? This should be repeated with shATAD3B PINK1 KO HeLa cells in the DMSO control.

6) Figure 4C: Why were cells treated with OA when it was shown before that ATAD3B promotes mitophagy independent from PINK1? One should repeat this IP with either H2O2 or 3-NPA since for those treatments ATAD3B was shown to promote mitophagy. OA is completely irrelevant here.

7) Figure 5: (B-D) ATAD3B is degraded in the same manner as TOM20 regardless of cell line and treatment and behaves clearly not as the authors claim like Tim23 with DMSO treatment. Thus, their hypothesis of a change in localization of ATAD3B in response to oxidative damage cannot be drawn from these results. Further H2O2 treatment in (D) reveals that mitochondria are more susceptible to digestion since here 10 µg/ml are already sufficient for degradation of the outer membrane (Tom20 and ATAD3B) in contrast to (C) where 30 µg/ml are needed. This leads to the conclusion that the outer membrane might be already damaged / ruptured / leaky due to the

treatment and thereby LIR of ATAD3B might get exposed. Thus, the authors should include experiments to prove the intactness of the membrane upon this treatment. (F) The mentioned decrease in ATAD3 oligomers is not really evident from the data provided. Please repeat this experiment. Overall, these data do not support the hypothesis that H2O2 treatment results in localization change of ATAD3B and reduced interaction with ATAD3A as claimed by the authors.
8) Figure 6C: The decrease for ATAD3B in 3243A>G comparing 100% WT and 90% mutated mtDNA is not as prominent as it was described by the authors, since WT ATAD3B seems generally to be quite low in expression compared to ATAD3A and normally patients do not have 90% of mutated mtDNA making the difference even smaller.
9) The suggested model shown in Figure 6I is mostly derived from suggestions without proper experimental validation since no clear localization change was visible in the provided data and loss of oligomer formation upon H2O2 treatment was not detected via immunoblot but only upon loss of mitochondrial DNA by ddC. Further experiments revealing a change of interactions upon oxidative stress between ATAD3A and B should be performed, e.g. IPs after H2O2 treatment. Further, their model shows possible cysteine bridges between those isoforms (this should be described in the figure legend), thus experiments should be performed under native as well as denaturing conditions.
10) Figure S3: It would be good to include the WT control cell line with H2O2 treatment to see if mitophagy levels can be fully or only partly reestablished upon ATAD3B-Flag expression.
11) Figure S1E: Why is only for HEK293T cells shown that nDNA is not susceptible to the treatment? The authors should add this control for MEFs and HeLa cells as well.
12) In the discussion it is mentioned that ATAD3B is activated due to decreased membrane potential, however this has not been shown in this study.
Minor comments
Generally, this report contains many spelling mistakes, inconsistency in abbreviations such as hr or h, space characters between numbers and units are missing and the report needs linguistic revision (e.g. usage of connectors and misleading formulations, -s ending for verbs of the 3rd person singular).
In Figure 1 E and Figure S5 depicted wavelengths indicate 448 nm and 552 nm, which is inconsistent with the figure legend saying cells were imaged with 458 and 561 nm laser excitation.
Figure 3: spelling error: mito instead of mtio
Figure 4 A: The IgG lane is not explained in the figure legend. Was IgG coupled onto the beads instead of antiATAD3 as control?
Figure 4 E: The ATAD3A sequence from 594-617 should be shown as well to illustrate that this isoform does not contain a LIR-3.
Figure S1: The authors do not specify which PCR products are actually shown in Figure S1 A, neither in the figure description nor in the figure itself. Are these the amplicons of the three different cell lines HEK293T, HeLa and MEFs? Furthermore, they misspelled mtDNA products.
Figure 6 F: It is not explained how the quantification was measured e.g. how many replicates were used for analysis.
Figure S1 G + S6 C are missing an explanation of what the arrows are depicting.
Figure S7 A: Domain Abbreviations should be explained in figure legend.

Referee #2:

This manuscript entitled "ATAD3B Mediates the Clearance of Oxidative Stress-induced Damaged mtDNA by Acting as a Mitophagy Receptor" by Li Shu et al. found that ATAD3B is the mitophagy receptor that clear damaged mtDNA. They showed that ATAD3B binds to LC3 via its LIR motif. Under oxidative condition, the C terminal of ATAD3B is exposed to cytosolic face and the LIR motif recruits LC3 to initiate mitophagy. Further they showed that m.3243A>G mtDNA mutation induced

oxidative stress and ATAD3B expression facilitate clearance of damaged mtDNA.

This is comprehensive study that covers from the identification of the mitophagy receptor to its physiological regulation. Data are mostly clean and reliable. The logic is straight forward and well documented. It is suitable for publication in the EMBO journal.

This is comprehensive study that covers from the identification of the mitophagy receptor to its physiological regulation. Data are mostly clean and reliable. The logic is straight forward and well documented. It is suitable for publication in the EMBO journal.

Referee #3:

This is an interesting report by Shu and colleagues who identified ATAD3B as a novel mitophagy receptor that recruits LC3 under conditions of oxidative stress-induced mitochondrial DNA (mtDNA) damage to promote the clearance of affected mitochondria. The authors further showed that the pathway is specific to the ATAD3B isoform (i.e. not ATAD3A) and is independent of PINK1/Parkin. Under normal conditions, the LC3 binding region of ATAD3B is kept within the mitochondrial inner membrane space via its hetero-oligomerization with ATAD3A. Oxidative stress compromises the formation of the ATAD3A/3B oligomerization leading to the exposure of the ATAD3B LC3 binding motif at the outer mitochondrial membrane that induces mitophagy. Finally, the authors showed that ATAD3B exhibits reduced expression in cell models of MELAS. Importantly, re-expression of ATAD3B facilitates the clearance of mtDNA mutation in these cells. Taken together, these findings help clarifies the current gaps in knowledge surrounding how damaged mtDNA triggers mitophagy and provides a potential mechanism underlying this process.

Notwithstanding the above, I do have some comments/suggestions for the author's consideration, as listed below.

1. The authors showed that ATAD3B KO Hela cells exhibit reduced 3-NPA-induced mitophagy. It would be interesting to examine the effects of ATAD3A KO cells in this context, i.e. whether mitophagy would be enhanced under basal or 3-NPA-treated condition without ATAD3A to localize the LC3 binding region of ATAD3B in the inner mitochondrial space.

2. Curiously, the proteinase K analysis shows that ATAD3B proteolysis mimics Tom20 more than Tim23 under basal condition, which is contrary to what the authors have stated. This is an important issue as it impacts on the author's proposed model of ATAD3B's mechanism of action.

3. The decrease in oligomerization in Fig. 5F following hydrogen peroxide treatment is quite modest to result in overt mitophagy. Further, given the robust expression of ATAD3A relative to ATAD3B, it is unclear how the mechanism really works since there will always be a mass action by ATAD3A.

4. Related to the above, the authors proposed that hetero-oligomerization of the ATAD3 proteins are impaired due to ROS increasing cysteine oxidation of ATAD3B protein. The burden of proof is needed here.

5. Would the addition of anti-oxidants promote the hetero-oligomerization of ATAD3A/3B under basal and oxidative stress conditions?

6. Is ATAD3B also involved in hypoxia-induced mitophagy, which is known to be associated with oxidative stress?

7. Minor: S1D showing no apparent difference in mtDNA damage in treated vs control MEFs that have undergone a washout is quite confusing. The authors later stated that MEFs lacks ATAD3B. Suggest for authors to explain this upon discussing S1D or move S1D to later section.

We sincerely thank the reviewers for their valuable comments and constructive criticisms, which were of great help in revising the manuscript. According to the reviewers' comments and suggestions, the revised manuscript has been systematically improved with new data and additional interpretations. Reviewers' comments are responded point-by-point as bellow. Reviewers' points are underlined and italic for easier reference. Descriptions for all newly performed experimental results and other changes are highlighted in blue in the revised manuscript.

Referee #1:

Manuscript summary

Shu et al. report the identification of a novel mitophagy receptor, ATAD3B, that mediates the clearance of mitochondria upon oxidative stressed-induced mtDNA damage. Firstly, the authors show that oxidative stressors, 3-NPA and H2O2 respectively, lead to irreparable mtDNA damage and provide evidence that the autophagy machinery is necessary to clear mtDNA damage by use of stable ATG5 knockdown cell lines. In order to assess possible regulators/initiators of mtDNA damage induced mitophagy, stable knockdown cell lines, targeting different mtDNA-binding or associated proteins, were compared in their efficiency to perform mitophagy using mito-Keima assays as readout. They identified ATAD3 as necessary factor for mitophagy events and state that isoform B promotes mitophagy in a PINK1-independent manner by performing mitophagy assays in PINK1 KO background. Further, they showed a direct interaction between ATAD3B and LC3 via its C-terminal LIR motif which is lacking in the 3A isoform. Next, Shu and colleagues claim that mtDNA damage induces a change of localization of ATAD3B from intermembrane space to outer mitochondrial membrane (OMM) by performing colocalization immunofluorescence stainings with OMM protein TOM20. Further, they analyzed the localization by gradual digestion of isolated mitochondria with proteinase K and analysis of the protein content via immunoblot. Their model implies that oxidative stress leading to DNA damage results in translocation to the outer membrane where the LIR region is getting accessible for LC3 binding and recruitment of the autophagy machinery. Furthermore, they state that ATAD3A and B undergo less oligomer formation upon oxidative stress. Finally, they could show that the pathogenic point mutation 3243 A>G, which causes MELAS, induces oxidative stress and that MELAS patient-derived fibroblasts hardly express ATAD3B. Reconstitution experiments with ATAD3B-Flag but not ATAD3A-Flag

resulted in clearance of mutated mtDNA showing the protective role of ATAD3B not only under chemically induced but also pathophysiological oxidative stress.

Major comments

1) The authors should explain how the frequency of mtDNA damage, which is shown in Figure 1 B and S1 B is actually calculated from the mtDNA amplification data. Further, in material and methods section it is not clear what is meant by "1/2 control was necessary for loading error".

We greatly appreciate the reviewer's comments. We used the quantitative PCR (qPCR) assay to measure mtDNA damage. The qPCR assay of DNA damage is based on the principle that many kinds of DNA lesions (including mutations and deletions) can block or slow down the progression of DNA polymerase. Therefore, if equal amounts of DNA from differently treated samples (DMSO, 3-NPA, or H_2O_2 treated cells in our manuscript) are qPCR amplified under identical conditions, DNA with more lesions will amplify to a less extent than fewer damaged DNA. DNA damage can be expressed in terms of lesions per kilobase (kb) mathematically by assuming a Poisson distribution of lesions. In our manuscript, the quantification of DNA damage was performed according to the previous reports (Hunter et al, 2010; Santos et al, 2002; Zhu & Coffman, 2017). DNA (mtDNA or nDNA) damage was quantified by comparing the relative efficiency of amplification of large DNA fragments (10 kb for mouse mtDNA, 6.6 kb for mouse nDNA, 8.9 kb for human mtDNA, 12.2 kb for human nDNA) of DNA from H_2O_2- and 3-NPA-treated samples to those of controls, and normalizing this to the amplification of smaller (<250bp) fragments, which have a statistically negligible likelihood of containing damaged bases. The resulting values are converted to relative lesion frequencies per 10kb DNA (mtDNA) (Figure 1B and Appendix Figure S1B of the revised manuscript) by application of the Poisson distribution (lesions/amplicon = −ln(At /Ao); where At is the amplification of treated samples and Ao represents the amplification of untreated controls).

Because the optimal number of PCR cycles to run is dependent on the initial amount of DNA, we performed a PCR cycle test using various amounts of initial DNA from control samples (untreated or non-damaged) to determine quantitative conditions. The optimal number of cycles over which a "1/2 control" amount of template reduces amplification to ~50% was identified. We then run PCR using 1/2 control as quality control.

We have added the description in the "Materials and Method" section of the revised manuscript.

References

Hunter SE, Jung D, Di Giulio RT, Meyer JN (2010) The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number. Methods (San Diego, Calif) 51: 444-451

Santos JH, Mandavilli BS, Van Houten B (2002) Measuring oxidative mtDNA damage and repair using quantitative PCR. Methods in molecular biology (Clifton, NJ) 197: 159-176

Zhu S, Coffman JA (2017) Simple and fast quantification of DNA damage by real-time PCR, and its application to nuclear and mitochondrial DNA from multiple tissues of aging zebrafish. BMC research notes 10: 269

2) It is not clear how the fluorescence intensity from immunofluorescence stainings was quantified since it is neither explained in the materials and methods section nor in figure legends. This information should be included: Was the fluorescence intensity of the whole image quantified or only prominent dots or just the enlarged sections? Also, it is not always mentioned in the figure legend how many replicates were used for the quantification e.g. Figure S3 C+E

We appreciate the reviewer's comments and suggestions. The fluorescence intensity from immunofluorescence staining or fluorescent images were quantified using ImageJ software, and the data were further analyzed by Microsoft Excel and GraphPad Prism 8. In detail, the mito-Keima fluorescence intensity of cells in the whole image (Figures 1E, 2B, 3A, EV1C, EV1E, EV1G, EV5A, EV5E, and Appendix Figure S4A of the revised manuscript) and the 8-oxo-dG fluorescence intensity of cells (or selected cells) in the whole image (Figures 1C, 2D, 3D, 3F, 3H, 3G, EV2A and EV2C of the revised manuscript) were quantified, and 20 cells (from 3-10 images) were randomly selected for quantification in each experiment, and at least 3 experiments (replicates) were performed. As suggested by the reviewer, we have added a detailed description in the "Materials and Methods" section, and added the number of replicates in the related figure legends of the revised manuscript.

3) For some cell lines used in this study, no validation is shown. Immunoblot analysis for prove of proper stable knockdown should be added for following cell lines:

a. Figure 2A: shLONP1, shSSBP1, shPEO1, shPOLG2, shPOLRMT, shTFAM, shATAD3, shATG5 HeLa

b. Figure 3A: PINK1 KO shATAD3B HeLa

According to the reviewer's suggestions, we have added the immunoblot analysis or quantitative PCR analysis (due to lack of antibody) to prove the proper stable knockdown of the following cell lines in the revised manuscript: a. shLONP1, shSSBP1, shPEO1, shPOLG2,

shPOLRMT, shTFAM, shATAD3, shATG5 HeLa (Appendix Figure S2 of the revised manuscript).

b. Figure 3A: PINK1 KO shATAD3B HeLa (Appendix Figure S4F of the revised manuscript).

4) Figure 1 G hardly shows colocalization events between GFP-LC3 and DNA/TOM20. Thus, one cannot state that these treatments induce specifically mitophagy, since the increase of GFP-LC3 could be also a general increase in autophagy. This experiment should be repeated with a quantification of colocalizing punctae.

We agree with the reviewer's comment. As suggested by the reviewer, we repeated the experiments, and provided the new images (showing more colocalization between GFP-LC3 and mtDNA/TOM20) and the quantification of colocalization between GFP-LC3 and mtDNA/TOM20 in Figures 1G and 1H of the revised manuscript.

5) Figure 3: Figure 3H and J are not describing the antibody used for the first staining panel. The figure legend says anti-Flag antibody but since PINK1 KO cells without Flag-tagged reconstitution were used, this seems not plausible. Further, in the DMSO treated cells no shATAD3B PINK1 KO HeLa cells are depicted, why? This should be repeated with shATAD3B PINK1 KO HeLa cells in the DMSO control.

We are sorry for the unclear or incorrect description in the figure legends of 3H and 3J. We infected the control cells with a nuclear localized GFP tag (H2B-GFP) to distinguish shATAD3B in PINK1 KO HeLa cells. Then control (shRNA vector expressing H2B-GFP) and shATAD3B (no GFP) were mixed (1:1) and cultured on a coverslip, which ensures that control and shATAD3B cells can be exposed to the same conditions during H_2O_2 (or DMSO, or 3-NPA) treatment, immunostaining and imaging process. Because the fluorescent intensity of 8-oxo-dG is very weak under DMSO treatment (Figures 3H and 3J), leading to shATAD3B cells being invisible although they actually existed. According to the reviewer's suggestions, we repeated the experiments, we mixed the control (H2B-GFP-labeled) and shATAD3B (no H2B-GFP) in a coverslip and treated with DMSO, 3-NPA or H_2O_2 for 2 h. Next, cells in the coverslip were washed with PBS and incubated with the medium for an additional 1 h (washout-1 h), and cells were then fixed and stained with anti-8-oxo-dG antibody and DAPI, and imaged by confocal microscope. We provided the new images (Figures 3H and 3J of the revised manuscript) in the revised manuscript, moreover, we circled the control and shATAD3B cells with the white dotted

line in the images, respectively.

6) Figure 4C: Why were cells treated with OA when it was shown before that ATAD3B promotes mitophagy independent from PINK1? One should repeat this IP with either H2O2 or 3-NPA since for those treatments ATAD3B was shown to promote mitophagy. OA is completely irrelevant here.

We sincerely thank the reviewer for the comments and agree with the reviewer's opinion that OA is completely irrelevant here. As suggested by the reviewer, we performed GST-pull down assay with H_2O_2-treated cell lysates. Western blotting analysis revealed that GST-ATAD3B(265-648aa) binds to LC3B-II, but GST, GST-ATAD3A(1-294aa), GST-ATAD3A(313-586aa) or GST-ATAD3B(1-246aa) fails to interact with LC3B-II. The new data were provided in Figure 4C of the revised manuscript.

7) Figure 5: (B-D) ATAD3B is degraded in the same manner as TOM20 regardless of cell line and treatment and behaves clearly not as the authors claim like Tim23 with DMSO treatment. Thus, their hypothesis of a change in localization of ATAD3B in response to oxidative damage cannot be drawn from these results. Further H2O2 treatment in (D) reveals that mitochondria are more susceptible to digestion since here 10 µg/ml are already sufficient for degradation of the outer membrane (Tom20 and ATAD3B) in contrast to (C) where 30 µg/ml are needed. This leads to the conclusion that the outer membrane might be already damaged / ruptured / leaky due to the treatment and thereby LIR of ATAD3B might get exposed. Thus, the authors should include experiments to prove the intactness of the membrane upon this treatment. (F) The mentioned decrease in ATAD3 oligomers is not really evident from the data provided. Please repeat this experiment. Overall, these data do not support the hypothesis that H2O2 treatment results in localization change of ATAD3B and reduced interaction with ATAD3A as claimed by the authors.

We are grateful to the reviewer for her/his and thoughtful critiques and constructive suggestions. According to the reviewer's comments, we repeated the proteinase K digestion assay using the changed concertation and temperature, we used low concertations (0, 2, 5, 10, 15, or 20 µg/ml) of proteinase K and performed experiments on ice (37°C in the previous manuscript). We found that proteinase K digested Tom20 but not ATAD3A, ATAD3B, Tim23 and HSP60 under normal conditions (DMSO treatment) (Figures 5B and 5C of the revised manuscript), indicating that

mitochondrial outer membrane is still not damaged/ruptured/leaky and Tim23 (mitochondrial inner membrane space protein), ATAD3A, ATAD3B and HSP60 (mitochondrial matrix protein) are not exposed to proteinase K. In addition, Western bolt analysis revealed that under normal conditions (DMSO treatment), the proteolysis of ATAD3B and ATAD3A was similar to that of Tim23 (IMS protein) and HSP60 (mitochondrial matrix protein) but not Tom20 (OMM protein) in 293T cells (Figures 5B and 5C of the revised manuscript), suggesting that ATAD3B localizes at the IMS or mitochondrial matrix but not OMM. However, upon H_2O_2 treatment, the proteolysis of ATAD3B but not ATAD3A was similar to that of Tom20 (Figures 5D and 5E of the revised manuscript), indicating that ATAD3B localization is changed to mitochondrial outer membrane upon H_2O_2 treatment. Also, as suggested by the reviewer, we tried to perform TEM analysis to test the integrity of mitochondria, however, the purified mitochondria are more vulnerable to EM fixation reagents, lots of purified mitochondria without proteinase K treatment were still damaged, so the TEM experiment is not suitable for testing proteinase K-treated mitochondria.

In addition, the decrease of ATAD3 oligomers in H_2O_2-treated samples was not really evident in the previous manuscript because the loading control (Tom40 and HSP60) in H_2O_2-treated samples is more than that in DMSO-treated sample. According to the reviewer's suggestion, we repeated the experiment about ATAD3 oligomers and performed Western blotting analysis using similar levels of loading control. 293T cells were treated with DMSO, NAC (N-acetyl-L-cysteine, ROS scavenger), H_2O_2, or H_2O_2 plus NAC for 2 h, and purified mitochondria were used for crosslinking. Compared with control or H_2O_2 plus NAC treatment, H_2O_2 treatment led to a remarkable decrease of ATAD3 oligomers (Figures 5G and 5H of the revised manuscript), indicating that the interaction between ATAD3B and ATAD3A is reduced upon H_2O_2 treatment. Additionally, the BN-PAGE assay revealed that ATAD3 complexes (containing ATAD3 oligomers) were also reduced in H_2O_2 treated cells (Figure EV4G of the revised manuscript). Moreover, we performed co-IP assay to test the interaction between ATAD3B and ATAD3A in DMSO- or H_2O_2-treated cells. After H_2O_2-treatment, the interaction between ATAD3B and ATAD3A was markedly reduced (Figures EV4E and EV4F of the revised manuscript).

8) Figure 6C: The decrease for ATAD3B in 3243A>G comparing 100% WT and 90% mutated mtDNA is not as prominent as it was described by the authors, since WT ATAD3B seems generally to be quite low in expression compared to ATAD3A and normally patients do not have 90% of mutated mtDNA making the difference even smaller.

We thank the reviewer for his/her comments. We repeated the Western blotting analysis and compared the expression of ATAD3B in WT and mutated cells containing 90% mutated (3243A>G) mtDNA. Compared to WT, mutated cells showed a remarkably reduced ATAD3B, and ATAD3B is undetectable even in Western blot with long exposure (Figure 6C of the revised manuscript). In addition, compared to WT fibroblasts, MELES patient-derived fibroblasts (less than 90% of mtDNA is mutated) also showed a remarkable reduction of ATAD3B (undetectable) (Figure 6D of the revised manuscript). Also, we admit that the expression of ATAD3B is lower than that of ATAD3A. However, the role of ATAD3B in regulating mitophagy under oxidative stress is little related to the level of ATAD3A since ATAD3A does not promote mitophagy upon oxidative stress. In addition, the low expression of ATAD3B is probably due to the fact that the level is sufficient to promote mitophay under oxidative stress and the high expression of ATAD3B may impair the functions of ATAD3A, which plays an important role in the maintenance of mitochondrial structure and functions (Baudier, 2018; Dorison et al, 2020; Peralta et al, 2018).

References

Baudier J (2018) ATAD3 proteins: brokers of a mitochondria-endoplasmic reticulum connection in mammalian cells. *Biological reviews of the Cambridge Philosophical Society* 93: 827-844

Dorison N, Gaignard P, Bayot A, Gelot A, Becker PH, Fourati S, Lebigot E, Charles P, Wai T, Therond P *et al* (2020) Mitochondrial dysfunction caused by novel ATAD3A mutations. *Molecular genetics and metabolism*

Peralta S, Goffart S, Williams SL, Diaz F, Garcia S, Nissanka N, Area-Gomez E, Pohjoismäki J, Moraes CT (2018) ATAD3 controls mitochondrial cristae structure in mouse muscle, influencing mtDNA replication and cholesterol levels. *Journal of cell science* 131

9) The suggested model shown in Figure 6I is mostly derived from suggestions without proper experimental validation since no clear localization change was visible in the provided data and loss of oligomer formation upon H2O2 treatment was not detected via immunoblot but only upon loss of mitochondrial DNA by ddC. Further experiments revealing a change of interactions upon oxidative stress between ATAD3A and B should be performed, e.g. IPs after H2O2 treatment. Further, their model shows possible cysteine bridges between those isoforms

(this should be described in the figure legend), thus experiments should be performed under native as well as denaturing conditions.

According to the reviewer's comments, we re-performed proteinase K digestion assay (concentration and temperature were changed) and co-IP assay (H_2O_2-treated samples were added). Additionally, we performed Western blotting assay using DMSO or H_2O_2 treated samples under native (BN-PAGE) and denaturing (SDS-PAGE) conditions in the revised manuscript. The new data were provided in Figures EV4G of the revised manuscript. Proteinase K digestion assay reveals that the localization of ATAD3B but not ATAD3A is changed upon H_2O_2 treatment (Figures 5B-5E of the revised manuscript). Crosslinking, BN-PAGE and co-IP assays reveal that the interactions between ATAD3B and ATAD3A were reduced in response to H_2O_2 treatment (Figures 5G, 5H, and EV4E-EV4G of the revised manuscript). Additionally, according to the reviewer's suggestion, we added the description "cysteine-cysteine interaction may contribute to the formation of ATAD3B-ATAD3A hetero-oligomers" in the figure legend of Figure 6I of the revised manuscript.

10) Figure S3: It would be good to include the WT control cell line with H2O2 treatment to see if mitophagy levels can be fully or only partly reestablished upon ATAD3B-Flag expression.

According to the reviewer's suggestions, we repeated the experiments related to Figure S3 (Figure EV1 in the revised manuscript), and WT control cell line with H_2O_2 or 3-NPA treatment was included. WT, ATAD3 DKO, or ATAD3 DKO HeLa cells expressing ATAD3A-Flag or ATAD3B-Flag (all cells were stably expressing mito-Keima) were treated with DMSO, H_2O_2 or 3-NPA for 2 h, and then mitophagy was analyzed by confocal microscopy. Mito-Keima assay revealed that mitophagy levels can be fully reestablished upon ATAD3B-Flag expression in ATAD3 DKO cells (Figures EV1C-EV1F of the revised manuscript).

11) Figure S1E: Why is only for HEK293T cells shown that nDNA is not susceptible to the treatment? The authors should add this control for MEFs and HeLa cells as well.

As suggested by the reviewer, we added the control (nDNA) for HeLa and MEFs cells in the revised manuscript (Appendix Figures S1E and S1F of the revised manuscript).

12) In the discussion it is mentioned that ATAD3B is activated due to decreased membrane potential, however this has not been shown in this study.

According to the reviewer's comments, we tested the change of mitochondrial membrane potential using TMRM staining in DMSO-, H_2O_2- or FCCP-treated HeLa cells. In response to H_2O_2 treatment, mitochondrial membrane potential was slightly decreased (Appendix Figure S1I of the revised manuscript). Therefore, we deleted the statement that ATAD3B is activated due to decreased membrane potential, and changed the words "damaged mtDNA results in mitochondrial dysfunction, leading to increased ROS production and decreased mitochondrial membrane potential, causing activation of ATAD3B that subsequently induce mitophagy" to "damaged mtDNA results in mitochondrial dysfunction, leading to increased ROS production, which could cause the decreased interaction between ATAD3B and ATAD3A and the change of ATAD3B localization, then causing activation of ATAD3B that subsequently induce mitophagy" in the discussion section of the revised manuscript.

Minor comments

Generally, this report contains many spelling mistakes, inconsistency in abbreviations such as hr or h, space characters between numbers and units are missing and the report needs linguistic revision (e.g. usage of connectors and misleading formulations, -s ending for verbs of the 3rd person singular).

We appreciate the reviewer's comments and suggestions. According to the reviewer's suggestion, we consistently used "h" (hour) in the revised manuscript, and we tried our best to improve the grammar of the revised manuscript.

In Figure 1 E and Figure S5 depicted wavelengths indicate 448 nm and 552 nm, which is inconsistent with the figure legend saying cells were imaged with 458 and 561 nm laser excitation.

We are sorry for our type-errors, we corrected 448 nm and 552 nm into 458 nm and 561 nm in the image of Figure 1E and Appendix Figure S4A of the revised manuscript.

Figure 3: spelling error: mito instead of mtio

We thank the reviewer for his/her carefully reading. We have corrected this error in the revised manuscript.

Figure 4 A: The IgG lane is not explained in the figure legend. Was IgG coupled onto the beads instead of antiATAD3 as control?

Thank the reviewer's comments. We used IgG coupled-beads as the control for co-IP assay in Figure 4A, the new data showing IgG bands were provided in Figure 4A of the revised manuscript. Also, we explained this in the figure legend of Figure 4A of the revised manuscript.

Figure 4 E: The ATAD3A sequence from 594-617 should be shown as well to illustratethat this isoform does not contain a LIR-3.

Because ATAD3A just contains 586 amino acids (Figure EV4A and Appendix Figure S3A), the 594aa-617aa of ATAD3A is not exist and thus not shown. We have added this explanation in the figure legend of Figure 4E of the revised manuscript.

Figure S1: The authors do not specify which PCR products are actually shown in Figure S1 A, neither in the figure description nor in the figure itself. Are these the amplicons of the three different cell lines HEK293T, HeLa and MEFs? Furthermore, they misspelled mtDNA products.

We appreciate the reviewer's comments and suggestions. As suggested by the reviewer, we provided a new image showing the PCR products of mtDNA and nDNA of HEK293, HeLa and MEFs in Appendix Figure S1A of revised manuscript. In addition, thanks for the reviewer's careful reading and we have corrected the misspelling in the revised manuscript.

Figure 6 F: It is not explained how the quantification was measured e.g. how many replicates were used for analysis.

We performed quantitative real-time PCR (qRT-PCR) using the TaqMan probe to evaluate the percentage of mutated G in MELAS fibroblast or 90% 3243A>G cell lines, and at least 3 independent experiments were performed. The detection of mtDNA 3243G mutation level was performed as previously described (Rong et al, 2018) where a probe special for mt.3243G (mutant) was labeled at the 5' end with 6-carboxyfluorescein (6-FAM) and at the 3' end with a minor groove binding (MGB) moiety that increases the melting temperature (Tm) of the probe and stabilizes probe-target hybrids. The qRT-PCR data were then normalized to mtDNA copy number. According to the reviewer's comments, we added the description in the section of "Materials and Methods", and provided the number of replicates in the figure legend of Figure 6F of the revised manuscript.

Reference

Rong E, Wang H, Hao S, Fu Y, Ma Y (2018) Heteroplasmy Detection of Mitochondrial DNA A3243G Mutation Using Quantitative Real-Time PCR Assay Based on TaqMan-MGB Probes. *BioMed Research International* 2018: 1286480

Figure S1 G + S6 C are missing an explanation of what the arrows are depicting.

Thanks the reviewer's comments. The red arrows indicate autophagosome, and the yellow arrows indicate the mitochondria in S1G (Appendix Figure S1H of the revised manuscript), and arrows in S6C (Figure EV3C of the revised manuscript) indicates the LC3 punta colocalizing or contacting with mitochondria. As suggested by the reviewer, we have added the explanation in the figure legends of Figures 4G, EV3C, and Appendix Figure S1H of the revised manuscript.

Figure S7A: Domain Abbreviations should be explained in figure legend.

As suggested by the reviewer, we have added the explanation in the figure legend of Figure EV4A of the revised manuscript.

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Referee #2:

This manuscript entitled "ATAD3B Mediates the Clearance of Oxidative Stress-induced Damaged mtDNA by Acting as a Mitophagy Receptor" by Li Shu et al. found that ATAD3B is the mitophagy receptor that clear damaged mtDNA. They showed that ATAD3B binds to LC3 via its LIR motif. Under oxidative condition, the C terminal of ATAD3B is exposed to cytosolic face and the LIR motif recruits LC3 to initiate mitophagy. Further they showed that m.3243A>G mtDNA mutation induced oxidative stress and ATAD3B expression facilitate clearance of damaged mtDNA.

This is comprehensive study that covers from the identification of the mitophagy receptor to its physiological regulation. Data are mostly clean and reliable. The logic is straight forward and well documented. It is suitable for publication in the EMBO journal.

We sincerely thank the reviewer for his/her comments. Descriptions for all newly performed experimental results and other changes are highlighted in blue in the revised manuscript.

Referee #3:

This is an interesting report by Shu and colleagues who identified ATAD3B as a novel mitophagy receptor that recruits LC3 under conditions of oxidative stress-induced mitochondrial DNA (mtDNA) damage to promote the clearance of affected mitochondria. The authors further showed that the pathway is specific to the ATAD3B isoform (i.e. not ATAD3A) and is independent of PINK1/Parkin. Under normal conditions, the LC3 binding region of ATAD3B is kept within the mitochondrial inner membrane space via its hetero-oligomerization with ATAD3A. Oxidative stress compromises the formation of the ATAD3A/3B oligomerization leading to the exposure of the ATAD3B LC3 binding motif at the outer mitochondrial membrane that induces mitophagy. Finally, the authors showed that ATAD3B exhibits reduced expression in cell models of MELAS. Importantly, re-expression of ATAD3B facilitates the clearance of mtDNA mutation in these cells. Taken together, these findings help clarifies the current gaps in knowledge surrounding how damaged mtDNA triggers mitophagy and provides a potential mechanism underlying this process.

Notwithstanding the above, I do have some comments/suggestions for the author's consideration, as listed below.

1. The authors showed that ATAD3B KO Hela cells exhibit reduced 3-NPA-induced mitophagy. It would be interesting to examine the effects of ATAD3A KO cells in this context, i.e. whether mitophagy would be enhanced under basal or 3-NPA-treated condition without ATAD3A to localize the LC3 binding region of ATAD3B in the inner mitochondrial space.

We greatly appreciate the reviewer's constructive comments and suggestions. According to the reviewer's suggestions, we used the mito-Keima assay to evaluate mitophagy in WT (control) and ATAD3A KO HeLa cells treated with DMSO, H_2O_2, or 3-NPA. ATAD3A KO exhibited a slightly increased mitophagy, moreover, ATAD3A KO remarkably promoted H_2O_2-, or 3-NPA-induced mitophagy (Figures EV5A and EV5B of the revised manuscript). ATAD3A KO caused a slight mitophagy under basal probably because ATAD3A depletion (or dysfunction) itself leads to mitochondrial dysfunction (Baudier, 2018; Dorison et al, 2020; Peralta et al, 2018), which may induce slightly mitophagy. Also, ATAD3A KO remarkably promoted H_2O_2-, or 3-NPA-induced mitophagy may due to the increased mitochondrial dysfunction and the increased ATAD3B localizing at the mitochondrial outer membrane. It should be noted that FUNDC1 and other mitophagy receptors, which locates at the mitochondrial outer membrane, do not induce

mitophagy under normal conditions (Liu *et al.*, 2014). Therefore, ATAD3B localizing at mitochondrial outer membrane caused by ATAD3A depletion may have little effect on inducing mitophagy under normal conditions, certain stimuli, such as H_2O_2 or 3-NPA, is required for ATAD3B in promoting mitophagy. We have added these data and discussed the related issues in the revised manuscript.

References

Baudier J (2018) ATAD3 proteins: brokers of a mitochondria-endoplasmic reticulum connection in mammalian cells. *Biological reviews of the Cambridge Philosophical Society* 93: 827-844

Dorison N, Gaignard P, Bayot A, Gelot A, Becker PH, Fourati S, Lebigot E, Charles P, Wai T, Therond P *et al* (2020) Mitochondrial dysfunction caused by novel ATAD3A mutations. *Molecular genetics and metabolism*

Liu L, Sakakibara K, Chen Q, Okamoto K (2014) Receptor-mediated mitophagy in yeast and mammalian systems. Cell research 24: 787-795

Peralta S, Goffart S, Williams SL, Diaz F, Garcia S, Nissanka N, Area-Gomez E, Pohjoismäki J, Moraes CT (2018) ATAD3 controls mitochondrial cristae structure in mouse muscle, influencing mtDNA replication and cholesterol levels. *Journal of cell science* 131

2. Curiously, the proteinase K analysis shows that ATAD3B proteolysis mimics Tom20 more than Tim23 under basal condition, which is contrary to what the authors have stated. This is an important issue as it impacts on the author's proposed model of ATAD3B's mechanism of action.

We are grateful to the reviewer for her/his constructive and thoughtful critiques and suggestions. proteinase K has very high activity at 37°C, and it is difficult to handle the degradation of mitochondrial proteins in OMM (outer mitochondrial membrane) or IMM (inner mitochondrial membrane). To further support this proposed model, we repeated the proteinase K assay with changed the experimental conditions. We used the reduced concentrations of proteinase K and digested protein samples on ice instead of 37°C. Western blotting analysis revealed that ATAD3B proteolysis was more similar to Tim23 (IMM protein) than Tom20 (OMM protein) under basal condition, while ATAD3B proteolysis was similar to Tom20 after H_2O_2 treatment (Figures 5B-5E of the revised manuscript).

3. The decrease in oligomerization in Fig. 5F following hydrogen peroxide treatment is quite modest to result in overt mitophagy. Further, given the robust expression of ATAD3A relative to ATAD3B, it is unclear how the mechanism really works since there will always be a mass action by ATAD3A.

We appreciate the reviewer's comments and suggestions. The decrease of ATAD3 oligomers in H_2O_2 treated samples was not really evident in the previous manuscript because the loading control (Tom40 and HSP60) in H_2O_2-treated sample is more than that in DMSO-treated sample. According to the reviewer's comments, we repeated the experiment about ATAD3 oligomers and performed Western blotting analysis with the similar loading control. 293T cells were treated with DMSO, NAC (N-acetyl-L-cysteine, ROS scavenger), H_2O_2, or H_2O_2 plus NAC for 2 h, and purified mitochondria were used for crosslinking. Compared with control or H_2O_2 plus NAC treatment, H_2O_2 treatment led to a remarkable decrease of ATAD3 oligomers (Figures 5G and 5H of the revised manuscript), indicating that the interaction between ATAD3B and ATAD3A is reduced upon H_2O_2 treatment. Moreover, we performed co-IP assay to test the interaction between ATAD3B and ATAD3A in DMSO- or H_2O_2-treated cells. After H_2O_2-treatment, the interaction between ATAD3B and ATAD3A was markedly reduced (Figures EV4E and EV4F of the revised manuscript). In addition, ATAD3A plays an important role in the maintenance of mitochondrial structure and functions (Baudier, 2018; Dorison et al, 2020; Peralta et al, 2018). Although the expression of ATAD3A is markedly more than that of ATAD3B, ATAD3A binds to lots of mitochondrial proteins (Baudier, 2018; Dorison et al, 2020; Peralta et al, 2018), probably causing just part of (not all) ATAD3A to interact with ATAD3B, and some ATAD3A may always not bind to ATAD3B due to the impairment of some other ATAD3A-interacting proteins. Indeed, co-IP assay showed that just part of ATAD3A binds to ATAD3B-Flag (overexpressed, the level is similar to ATAD3A) under normal conditions (Figure EV5E of the revised manuscript). In addition, high expression of ATAD3B may impair the function of ATAD3A (Baudier, 2018), which may explain the low expression of ATAD3B in cells. We have discussed this issue in the revised manuscript.

References

Baudier J (2018) ATAD3 proteins: brokers of a mitochondria-endoplasmic reticulum connection in mammalian cells. *Biological reviews of the Cambridge Philosophical Society* 93: 827-844

Dorison N, Gaignard P, Bayot A, Gelot A, Becker PH, Fourati S, Lebigot E, Charles P, Wai T, Therond P *et al* (2020) Mitochondrial dysfunction caused by novel ATAD3A mutations. *Molecular genetics and metabolism*

Peralta S, Goffart S, Williams SL, Diaz F, Garcia S, Nissanka N, Area-Gomez E, Pohjoismäki J, Moraes CT (2018) ATAD3 controls mitochondrial cristae structure in mouse muscle, influencing mtDNA replication and cholesterol levels. *Journal of cell science* 131

4. Related to the above, the authors proposed that hetero-oligomerization of the ATAD3 proteins are impaired due to ROS increasing cysteine oxidation of ATAD3B protein. The burden of proof is needed here.

We appreciate the reviewer's comments. ATAD3A contains 27 cysteines (S) and ATAD3B contains 33 cysteines, so we previously presumed that ROS may change cysteine oxidation of ATAD3B and ATAD3A, thus impairing the interaction between ATAD3B and ATAD3A (self-oxidation of ATAD3A or ATAD3B may impair the ATAD3A-ATAD3B interaction), and causing the decreased hetero-oligomerization of the ATAD3 proteins. However, there are many challenges and limitations to detect oxidative modifications at cysteine and to determine the site and type of oxidative modification of cysteine (Alcock et al, 2018). Furthermore, another challenge is the short lifetime of some of these oxidative modifications of cysteine (Alcock et al, 2018). Also, in addition to cysteine oxidation, some other factors may also impair hetero-oligomerization of the ATAD3 proteins under H_2O_2 treatment. Therefore, according to the reviewer's comments, we restated that hetero-oligomerization of the ATAD3 proteins is impaired due to the decreased interaction between ATAD3B and ATAD3A under H_2O_2 treatment (Figures EV4E and EV4F of the revised manuscript) and deleted the related statement about cysteine oxidation in the revised manuscript.

Reference

Alcock LJ, Perkins MV, Chalker JM (2018) Chemical methods for mapping cysteine oxidation. *Chemical Society reviews* 47: 231-268

5. Would the addition of anti-oxidants promote the hetero-oligomerization of ATAD3A/3B under basal and oxidative stress conditions?

According to the reviewer's suggestion, we repeated the experiment about ATAD3 oligomers and performed Western blotting analysis. 293T cells were treated with DMSO, NAC (N-acetyl-L-cysteine, ROS scavenger), H_2O_2, or H_2O_2 plus NAC for 2 h, and purified mitochondria were used for crosslinking. Compared with control or H_2O_2 plus NAC treatment, H_2O_2 treatment led to a remarkable decrease of ATAD3 hetero-oligomers (Figures 5G and 5H of the revised

manuscript), indicating that the interaction between ATAD3B and ATAD3A is reduced upon H_2O_2 treatment. In addition, under basal conditions (without H_2O_2 treatment), NAC treatment resulted in a slight increase (not significant) of ATAD3 hetero-oligomers (Figure 5G and 5H of the revised manuscript). Moreover, compared to H_2O_2 treatment, H_2O_2 plus NAC treatment led to remarkable increased of ATAD3 hetero-oligomers (Figure 5G and 5H of the revised manuscript), indicating that NAC (anti-oxidant) promotes the hetero-oligomerization of ATAD3A/3B under oxidative stress conditions.

6. Is ATAD3B also involved in hypoxia-induced mitophagy, which is known to be associated with oxidative stress?

According to the reviewer's suggestions, control, ATAD3B knockdown (shATAD3B), or ATAD3B-Flag-expressed HeLa cells stable expressing mito-Keima were cultured in normoxia or hypoxia (1% O_2) for 12 h. Cells were then used for analysis of mitophagy and mtROS (mitochondrial ROS). In response to hypoxia, mtROS production was greatly increased (Figures EV5C and EV5D of the revised manuscript), suggesting that hypoxia leads to oxidative stress in cells. Moreover, mito-Keima assay reveal that compared to control, ATAD3B knockdown led to a remarkable decrease of hypoxia-induced mitophagy, and ATAD3B-Flag expression caused a markedly increase of hypoxia-induced mitophagy (Figures EV5E and EV5F of the revised manuscript), suggesting that ATAD3B promotes hypoxia-induced mitophagy. We have added these data in the revised manuscript.

7. Minor: S1D showing no apparent difference in mtDNA damage in treated vs control MEFs that have undergone a washout is quite confusing. The authors later stated that MEFs lacks ATAD3B. Suggest for authors to explain this upon discussing S1D or move S1D to later section.

We appreciate the reviewer's comments and suggestions. According to the reviewer's suggestions, we put Figure S1D to be Figure EV2G in the revised manuscript.

1st Revision - Editorial Decision 17th Dec 2020

Thank you for submitting a revised version of your study. The manuscript has now been sent back to two of the original referees, whose comments are appended below.

As you will see, reviewer #3 finds that his/her criticisms have been sufficiently addressed and recommends the study for publication. However, referee #1 stresses that important controls are lacking in the experiments in which mitochondria were treated with proteinase K. In addition, this referee feels that the proposed model of ATAD3B acting as mitophagy receptor upon oxidative stress does not explain what triggers ATAD3B activation. Therefore, I ask you to include the controls requested by referee #1 and to carefully discuss the possible mechanism of ATAD3B activation in your system.

In addition, there are a few editorial issues concerning the text and the figures that I need you to solve before we can officially accept your manuscript for publication here.

Referee #1:

The authors nicely responded to the reviewer's comments and provided answers for most of the questions. The repetition of new experiments and addition of new data show more prominent the decrease in oligomerization of the ATAD complex upon oxidative stress. However, I still need to raise two critical points about the mitochondria proteinase K treatments:
Figure 5B-C: With these new experimental conditions that the authors chose, no proteolysis of ATAT3A as well as Tim23 and HSP60 is visible. Please include a control with higher proteinase K concentration to see the proteolysis of the inner mitochondrial part under DMSO and H2O2 treated conditions, otherwise you cannot claim that ATAD3B and ATAD3A underwent proteolysis similar to TIM23 with DMSO treatment, since no proteolysis happened at 20 µg/ml proteinase K treatment, yet.
Figure 5K: Please show the proteolysis of ATAD3B in ATAD3A KO cells with the new experimental conditions as well so that one can compare the proteolysis of ATAD3B between ATAD3A KO and H2O2 treatment condition.

Moreover, the proposed model of the authors, which claims that ATAD3B acts as mitophagy receptor upon oxidative stress, still leaves many questions open.
First, the authors did not elucidate the mechanism of action, in particular how H2O2 or 3-NPA treatment results in de-oligomerization of ATAD3A and ATAD3B. Second, they showed that change in localization of ATAD3B to the OMM is not sufficient to induce mitophagy without oxidative stress as shown for ATAD3A KO cells (Figure EV5 A, B). Thus, the proposed model lacks the actual trigger for ATAD3B activation.

Referee #3:

The authors have addressed the majority of my comments to my satisfaction. I am pleased to note the inclusion of additional experimental data in the revised manuscript. Overall, the current report represents a significantly improved version of the original one.

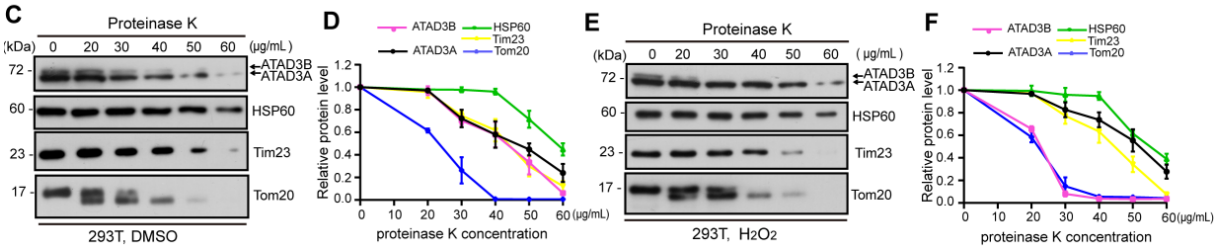
We sincerely thank the reviewers for their valuable comments and suggestions. According to the reviewers' comments and suggestions, the revised manuscript has been improved with new data and additional interpretations. Reviewers' comments are responded point-by-point as bellow. Reviewers' points are underlined and italic for easier reference. Descriptions for all newly performed experimental results and other changes are highlighted in red in the revised manuscript (EMBOJ-2020-106283R1).

Referee #1:

The authors nicely responded to the reviewer's comments and provided answers for most of the questions. The repetition of new experiments and addition of new data show more prominent the decrease in oligomerization of the ATAD complex upon oxidative stress. However, I still need to raise two critical points about the mitochondria proteinase K treatments:

Figure 5B-C: With these new experimental conditions that the authors chose, no proteolysis of ATAT3A as well as Tim23 and HSP60 is visible. Please include a control with higher proteinase K concentration to see the proteolysis of the inner mitochondrial part under DMSO and H2O2 treated conditions, otherwise you cannot claim that ATAD3B and ATAD3A underwent proteolysis similar to TIM23 with DMSO treatment, since no proteolysis happened at 20 µg/ml proteinase K treatment, yet.

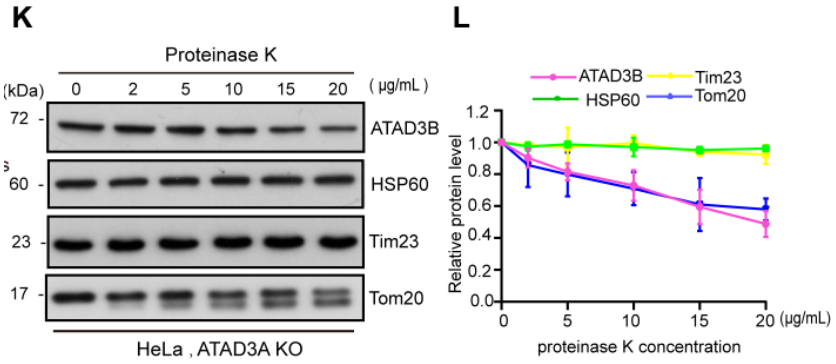
We greatly appreciate the reviewer's comments and suggestions. As suggested by the reviewer, we performed a proteinase K assay (control) with higher protease K concentrations (0, 20, 30, 40, 50, or 60 µg/ml) to analyze the proteolysis of the inner mitochondrial part under DMSO- and H_2O_2-treated conditions. After treatment with higher protease K concentrations (30, 40, 50, or 60 µg/ml), Tim23 (mitochondrial inner membrane space protein), ATAD3A or ATAD3B was proteolyzed under DMSO- and H_2O_2-treated conditions (Figure EV4C-4F). In addition, after treatment with 50 or 60 µg/ml protease K concentration, mitochondrial matrix protein HSP60 was proteolyzed under DMSO and H_2O_2 treated conditions (Figure EV4C-4F). We have provided these data in the revised manuscript (EMBOJ-2020-106283R1).



Figures EV4C-EV4F in the revised manuscript (EMBOJ-2020-106283R1).

Figure 5K: Please show the proteolysis of ATAD3B in ATAD3A KO cells with the new experimental conditions as well so that one can compare the proteolysis of ATAD3B between ATAD3A KO and H2O2 treatment condition.

According to the reviewer's suggestion, we performed a proteinase K assay with the new experimental conditions (0, 2, 5, 10, 15, and 20 μg/ml protease K) to analyze the proteolysis of ATAD3B in ATAD3A KO cells. ATAD3B and Tom20, but not Tim23 and HSP60, was proteolyzed by 5, 10, 15 and 20 μg/ml protease K in ATAD3A KO cells (Figures 5K and 5L of the revised manuscript), which is similar to the H_2O_2-treated condition (Figures 5D and 5E of the revised manuscript).



Figures 5K and 5L in the revised manuscript (EMBOJ-2020-106283R1).

Moreover, the proposed model of the authors, which claims that ATAD3B acts as mitophagy receptor upon oxidative stress, still leaves many questions open.

First, the authors did not elucidate the mechanism of action, in particular how H2O2 or 3-NPA treatment results in de-oligomerization of ATAD3A and ATAD3B. Second, they showed that change in localization of ATAD3B to the OMM is not sufficient to induce mitophagy without oxidative stress as shown for ATAD3A KO cells (Figure EV5 A, B). Thus, the proposed model lacks the actual trigger for ATAD3B activation.

We appreciate the reviewer's comments. To induce mitophagy, besides activating mitophagy receptor, autophagy still needs to be activated. For example, Fundc1 (a mitophagy receptor) locates at the mitochondrial outer membrane and doesn't induce mitophagy under normal condition (Liu et al, 2012; Liu et al, 2014); upon hypoxia, autophagy is activated, then fundc1 recruits LC3 to initiate mitophagy (Liu et al, 2012; Liu et al, 2014). Similarly, CCCP not only induces PINK1 translocating to the mitochondrial outer membrane, but also induces autophagy (Kwon et al, 2011; Narendra et al, 2010), both of which contribute to mitophagy. In our manuscript, ATAD3B binds to LC3B-II (Figures 4C and 4I). In normal conditions or ATAD3A KO cells, autophagy is not activated, and LC3B mainly exists in the form of LC3B-I (but not

LC3B-II). Upon oxidative stress (H_2O_2 or 3-NPA treatment), autophagy is activated (Gao, 2019), and LC3B mainly exists in the form of LC3B-II, which can be recruited by ATAD3B, leading to the initiation of mitophagy.

About how H2O2 or 3-NPA treatment results in de-oligomerization of ATAD3A and ATAD3B, we think that the protein structure or modification (such as phosphorylation) of ATAD3A and ATAD3B may be changed upon H_2O_2 or 3-NPA treatment, leading to de-oligomerization of ATAD3A and ATAD3B. We will further clarify this in our future study.

According to the reviewer's and editor's comments (editor's comments: "to carefully discuss the possible mechanism of ATAD3B activation in your system"), we have carefully discussed the possible mechanism of ATAD3B activation in the section of "Discussion" of the revised manuscript (EMBOJ-2020-106283R1).

References

Gao Q (2019) Oxidative Stress and Autophagy. *Advances in experimental medicine and biology* 1206: 179-198

Kwon KY, Viollet B, Yoo OJ (2011) CCCP induces autophagy in an AMPK-independent manner. *Biochemical and biophysical research communications* 416: 343-348

Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, Ma Q, Zhu C, Wang R, Qi W *et al* (2012) Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. *Nature cell biology* 14: 177-185

Liu L, Sakakibara K, Chen Q, Okamoto K (2014) Receptor-mediated mitophagy in yeast and mammalian systems. *Cell research* 24: 787-795

Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS biology* 8: e1000298

Referee #3:

The authors have addressed the majority of my comments to my satisfaction. I am pleased to note the inclusion of additional experimental data in the revised manuscript. Overall, the current report represents a significantly improved version of the original one.

We sincerely thank the reviewer for his/her comments.

2nd Revision - Editorial Decision 1st Feb 2021

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

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: Zhiyin Song
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-106283

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?	All the data were repeated at least three times. We have used at least triplicates in experiments in which statistics are shown.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	There were no animal sutdies in this manuscript.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the ananlysis.
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.	No steps were taken to minimize the effects of subjective bias, however, all samples were measured in biological triplicates to minimize experimental biases.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical were used justified.
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, standard deviations (s.d.)were shown where indicated.

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

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

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

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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).	All the antibodies and reagents are referenced appropriately.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines used in this study are listed appropriatly and tested for mycoplasma contamination.

* 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.	There are no animal studies in this manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

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