EMBO reports

ATAD1 inhibits hepatitis C virus infection by removing the viral TA-protein NS5B from mitochondria

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Transaction Report:

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Dear Prof. Li

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns that need to be addressed to strengthen your data and its pathophysiological relevance. The potential interference of the HA tag, the stability of NS5B in CHX chase experiments, the localization of NS5B in HCV-infected cells and the impact of ATAD1 KO on the infectious titer for several HCV isolates need to be investigated. It will also be important to test whether the absence of ATAD1 function has an impact on antiviral immunity via MAVS signalling and whether the deletion mutants of ATAD1 fold properly.

From the referee comments it is clear that a major revision will be required to substantiate your conclusions. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (May 13th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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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 form of your manuscript when it is ready.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this manuscript, Zhou et al. found that the AAA-ATPase ATAD1 in the mitochondrial outer membrane negatively regulates HCV infection by directing mitochondrial mislocalized NS5B proteins to proteasomal degradation. It has been well studied that ATAD1 (yeast Msp1) can extract mislocalized endoplasmic reticulum (ER) or peroxisomal tail-anchored (TA) proteins from the mitochondrial outer membrane in an ATP-dependent manner, thereby promoting their degradation by the proteasome or their re-targeting to the ER. The finding that such a function of Msp1/ATAD1 could also be involved in its antiviral activity is very interesting. I only have a few suggestions that should be considered by the authors.

Major points

1. in Fig. 3, Zhou et al. analyze the localization of EGFP-NS5B in WT and ATAD1KO cells, but we have questions about this data. First, the WT cells have a lower PCC of 0.59 and ATAD1KO cells have a lower PCC of 0.77 (Fig. 3A). I believe this is because there is a dot-like signal of EGFP-NS5B that does not co-localize with DeRed-Mito in WT. What is the origin of this dot-like signal? Also, why is this dot-like signal observed less frequently in ATAD1KO cells? Furthermore, are the vertical labels in the line profiles in Figure 3B not inverted between WT and ATAD1KO? This seems to differ from the description.

2. By comparing the amount of NS5B protein at steady state, Zhou et al. showed that NS5B is degraded in an ATAD1-dependent manner. However, if there is a difference in the amount of NS5B transcribed between WT and ATAD1KO, the comparison of steady-state levels is not accurate. Rather, it would be important to present the half-life of NS5B by cycloheximide (CHX) chase experiment. This should at least be discussed.

3. Zhou et al. used constructs with HA tag (HA-ATAD1) and FLAG tag (FLAG-NS5B) at the N-terminus of ATAD1 and NS5B, respectively, to examine the binding of ATAD1 and NS5B by immunoprecipitation experiments. I have some concerns as follows. First, since the transmembrane domain (TMD) of ATAD1 is required for localization to the mitochondrial membrane, I wondered if the N-terminal tagging would not interfere with ATAD1 localization. In Fig. 4A, Zhou et al. examined the subcellular localization of HA-ATAD1 by immunostaining, but I wondered whether signal of HA-ATAD1 was co-localized to mitochondrial markers. Second, Zhou et al. constructed a variant of NS5B deleted the putative TMD (NS5B-D21aa) and examined its binding to HA-ATAD1, but to me regarding the results in Fig. 4B as well as Figs. 5A and 5B, the mobility on SDS-PAGE looks the same for full length NS5B and NS5B-D21aa. This should at least be discussed. Third, In Fig. 4D, Zhou et al. use deletion mutants to examine the region of ATAD1 required for binding to NS5B, but I wondered if these deletion mutants of ATAD1 retain the tertiary and quaternary structures of native ATAD1. At the very least, by deleting the walker B, WD motif, and arginine finger of ATAD1, could it be that ATAD1 is destabilized?

Minor points
1. According to previous literature, NS5B is thought to have an RNA-dependent RNA polymerase domain at the N-terminus and a single TMD consisting of 21 amino acids at the C-terminus, taking the topology of a TA protein. However, the domain structure of NS5B and the putative location of the TMD are unclear in this manuscript. Please create a new figure to help the reader understanding.

Referee #2:

HCV infection is one of the leading reason of CHC world widely. Protease and polymerase inhibitors could effectively cure HCV infection in clinics, however, we are still lack of understanding of virus-host interaction. In this manuscript, Li and his colleagues utilized HCV cell culture model in vitro to examine the ATAD1 on HCV infection. They found that ATAD1 KO could dramatically enhance HCV infection, and mechanistic studies found that ATAD1 could interact with viral NS5B protein and then cause NS5B

degradation via proteasome pathway. Overall, this manuscript is well organized and the experiments were well controlled. I have three major questions for the authors to be addressed:
1. The authors should exclude other reasons which lead to enhanced HCV infection when ATAD1 KO. As ATAD1 is responsible for mitochondrial homeostasis, and MAVS localized at mitochondiria, is it possible that ATAD1 KO could impair the antiviral immunity leading to enhanced HCV infection?
2. According to the mechanism of ATAD1 in this study, the reviewer is curious whether ATAD1 function is conserved in other RNA viruses or even DNA viruses? The authors could use other RNA viruses, such as ZIKA or DENV to test the specificity.
3. the authors could further clarify the rationales to test ATAD1 on HCV infection. is there any relevance of ATAD1 in HCV pathogenesis? Is there any evidence that HCV infection impair ATAD1 function at mitochondria?

Referee #3:

Zhou et al identify the mitochondrial protein ATAD1 as a negative regulator of hepatitis C virus infection. The authors employ a variety of techniques (CRISPR/Cas9; high resolution microscopy) and different HCV isolates to examine how ATAD1 influences HCV replication. In particular, they find that ATAD1 removes and directs the HCV protein NS5A towards proteasomal degradation. Despite decades of research the live cycle of HCV and the role of various host factors remains incompletely understood. This study is therefore another piece in the puzzle. Overall the content of the manuscript is well structured and the experiments are performed at a very high quality (in particular the western blots). The authors might consider to address the following minor points to further improve the quality of their manuscript:
Major comments:
The authors mainly use core protein western blots as a readout for HCV infection and only provide infectious titers for a single HCV isolate (JFH1). They might consider to confirm reduction of infectious viral titers upon ATAD1 knockout for further HCV isolates.
Moreover, the authors could distinguish whether ATAD1 knockdown leads to a reduced number of infected cells or the same number of infected cells but with lower levels of the core protein (i.e. via immunofluorescence of the core protein).The authors may determine colocalization of NS5B and Mito in HCV-infected cells. The analysis of NS5B localization is mainly based on vector-based expression of NS5B.
There is a discrepancy of the PCC in different Figures, although the experiment is the same. The PCC of NS5B and dsredMito in Figure 2C is 0,75 and in Figures 3c it is 0,5.
Minor comments:
Figure 2A: The fragmentation of the mitochondria is hard to judge from a single image of one cell, the authors might explain more thoroughly in the text how they define and quantify mitochondria fragmentation.
Figure 2C: The coloring of the median line/error bars or symbols might be adapt (now its black on black).
Figure 3B: The panels for WT and ATAD1-KO seem mislabeled, given that for the KO cells a stronger co-localization is expected as seen in 3C.
Only Student's unpaired t-test was performed, this does not seem appropriate for all data sets here, since sometimes multiple comparisons were performed.
Line 382. The authors wrote that they "analyzed the colocalization by confocal microscopy" but only show a single image for each condition. Please provide more comprehensive analysis or rephrase.
Please proofread the whole manuscript. There are several grammar errors and typos (i.e. Fig S1E "fag" instead of "flag").

Comments from the Editor

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns that need to be addressed to strengthen your data and its pathophysiological relevance. The potential interference of the HA tag, the stability of NS5B in CHX chase experiments, the localization of NS5B in HCV-infected cells and the impact of ATAD1 KO on the infectious titer for several HCV isolates need to be investigated. It will also be important to test whether the absence of ATAD1 function has an impact on antiviral immunity via MAVS signaling and whether the deletion mutants of ATAD1 fold properly.

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Authors: We greatly appreciate the editor for processing our manuscript for review and giving your positive decision on this study. We have carefully considered the comments from the reviewers and revised the manuscript, or answered the questions/concerns in the point-by-point letter, to address the comments raised by the reviewers. Overall, we believe that the quality of the manuscript has been considerably improved and look forward to hearing from you for its publication in The EMBO Reports.

We have performed additional experiments and generated a significant amount of new data, and some of the original data was replaced with new data with better quality, in order to address the reviewers' comments. The corresponding revisions related to the new experimental date, reagents,

and descriptions are all highlighted in the text in RED color. The new data included: **Figure 1O-P, 2B-2E, Figure 3, Figure 4A-E, Figure 5A, 5C-D, Figure 6, Figure 7, Figure EV2D-E, Figure EV4, and Appendix Figures S1-S5.**

During revision, we got significant assistance for the structural analysis form Dr. Ziwen Wang and Dr. Song Gao, thus they are qualified as co-authors. Mr. Yuhao Yang contributed significantly during this study, plus additional contribution with performing key experiments during revision, therefore qualifying for co-first author. The first author, Qing Zhou, got support from her employer institution, thus affiliation No. 2 was added.

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports
****

Point-by-point responses

Referee #1:

In this manuscript, Zhou et al. found that the AAA-ATPase ATAD1 in the mitochondrial outer membrane negatively regulates HCV infection by directing mitochondrial mislocalized NS5B proteins to proteasomal degradation. It has been well studied that ATAD1 (yeast Msp1) can extract mislocalized endoplasmic reticulum (ER) or peroxisomal tail-anchored (TA) proteins from the mitochondrial outer membrane in an ATP-dependent manner, thereby promoting their degradation by the proteasome or their re-targeting to the ER. The finding that such a function of Msp1/ATAD1 could also be involved in its antiviral activity is very interesting. I only have a few suggestions that should be considered by the authors.

Authors: We thank this reviewer for your positive comments to our study. The involvement of ATAD1 in antiviral responses represents a novel role for this protein, in addition to its established functions. During the revision process, we also discovered that ATAD1 plays a role in regulating MAVS-mediated antiviral activity (as noted by another reviewer), although further investigation is needed to fully understand the underlying mechanism. Collectively, these findings provide new insights into the function of ATAD1.

Major points

1. in Figure 3, Zhou et al. analyze the localization of EGFP-NS5B in WT and ATAD1KO cells, but we have questions about this data. First, the WT cells have a lower PCC of 0.59 and ATAD1KO cells have a lower PCC of 0.77 (Figure 3A). I believe this is because there is a dot-like signal of EGFP-NS5B that does not co-localize with DeRed-Mito in WT. What is the origin of this dot-like signal? Also, why is this dot-like signal observed less frequently in ATAD1KO cells?

Authors: Thank you for pointing out this question. It has been reported that NS5B was localized to both mitochondrial and endoplasmic reticulum (ER) membrane (Chu *et al*, 2011; Ivashkina *et al*, 2002; Schmidt-Mende *et al*, 2001). Here, we have demonstrated that NS5B induced mitochondrial fragmentation and a portion of NS5B was localized to mitochondria (new **Figure 2B-E**), the fragmented mitochondria with EGFP-NS5B localization exhibited the dot-like signals of EGFP-NS5B. In addition to mitochondrial localization of NS5B, a fraction of NS5B could also localize to the ER membrane, which could be the origin of those dot-like signals that did not co-localize with DsRed-Mito. To verify this, we co-transfected WT and ATAD1^{KO} Huh7.5 cells with EGFP-NS5B and DsRed-Mito, and visualized the ER by immunostaining with anti-Calnexin (new **Figure 3**). The confocal images demonstrated that a fraction of EGFP-NS5B did not co-localized with DsRed-Mito on mitochondria but instead localized to the ER in both WT and ATAD1^{KO} Huh7.5 cells (solid box in Crop) (new **Figure 3A**).

Furthermore, the co-localization of NS5B and mitochondria, or NS5B and ER, were analyzed by Imaris 8.4 software and depicted in new **Figure 3B** and **3C.** The results demonstrated that a higher frequency of NS5B co-localization with mitochondria in ATAD1^{KO} Huh7.5 cells compared to WT Huh7.5 cells, thus resulting in an increased degree of EGFP-NS5B co-localized with DsRed-Mito

(new **Figure 3)**. As ATAD1 functioned to remove NS5B that was localized to mitochondria, therefore deficiency of ATAD1 ($ATAD1^{KO}$) could exhibit a higher level of mitochondrial localization for NS5B. Therefore, those dot-like signals outside the mitochondria become less frequent.

With new images taken, we have re-analyzed the PCC of EGFP-NS5B and mitochondria.

For the revisions corresponding to this question, please see Figure 3.

2. By comparing the amount of NS5B protein at steady state, Zhou et al. showed that NS5B is degraded in an ATAD1-dependent manner. However, if there is a difference in the amount of NS5B transcribed between WT and ATAD1KO, the comparison of steady-state levels is not accurate. Rather, it would be important to present the half-life of NS5B by cycloheximide (CHX) chase experiment. This should at least be discussed.

Authors: We agree with this comment and have performed additional experiments to demonstrate the degradation of NS5B by CHX chase assay. We have replaced the original Figure 5B with new data **Figure 5C, 5D** and **EV4C.** The CHX chase experiment obviously showed that NS5B was degraded in an ATAD1-dependent manner in both Huh7.5 and 293T cells.

In new **Figure 5C** and **5D**, we co-transfected 293T cells with Flag-NS5B and ATAD1-HA or vector-HA for 24 hours, and then the cells were treated with CHX for indicated duration. In new **Figure EV4C**, we transfected WT, $ATAD1^{KO}$ and $ATAD1^{KO+Res}$ Huh7.5 cells with Flag-NS5B for 24 hours, and then the cells were treated with CHX for indicated duration. The results from western blotting and the ratio of Flag:GAPDH (new **Figure EV4C**) results obviously indicated that the degradation of NS5B was in an ATAD1-dependent manner.

3. Zhou et al. used constructs with HA tag (HA-ATAD1) and FLAG tag (FLAG-NS5B) at the N-terminus of ATAD1 and NS5B, respectively, to examine the binding of ATAD1 and NS5B by immunoprecipitation experiments. I have some concerns as follows. First, since the transmembrane domain (TMD) of ATAD1 is required for localization to the mitochondrial membrane, I wondered if the N-terminal tagging would not interfere with ATAD1 localization. In Figure 4A, Zhou et al. examined the subcellular localization of HA-ATAD1 by immunostaining, but I wondered whether signal of HA-ATAD1 was co-localized to mitochondrial markers.

Authors: We appreciate these professional and valuable comments. To rule out whether HA tag at the N-terminus or the C-terminus impacts the localization of ATAD1, we performed co-localization of ATAD1-HA (HA tag at the C-terminus of ATAD1) and HA-ATAD1 (HA tag at the N-terminus) with mitochondria as well as ATAD1-HA and HA-ATAD1 with NS5B. We co-transfected Huh7.5 cells with HA-ATAD1 or ATAD1-HA with DsRed-Mito, captured the

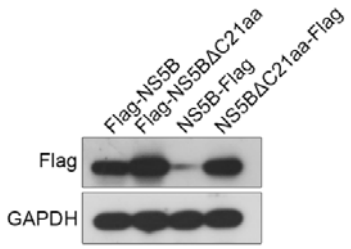
images using Zeiss LSM800, and analyzed their co-localization by analysis of Pearson correlation coefficient (PCC) using Imaris 8.4 software. The results demonstrated that the HA tag did not apparently impact the co-localization of ATAD1 with mitochondrial marker and the co-localization with NS5B. These data are present in new **Figure 4A-D**).

Second, Zhou et al. constructed a variant of NS5B deleted the putative TMD (NS5B-D21aa) and examined its binding to HA-ATAD1, but to me regarding the results in Figure 4B as well as Figs. 5A and 5B, the mobility on SDS-PAGE looks the same for full length NS5B and NS5B-D21aa. This should at least be discussed.

Authors: We understand the concern from this reviewer. Firstly, in order to exclude the potential influence of Flag tag position, we generated NS5B-Flag and NS5BΔC21aa-Flag plasmids (Flag tag at the C-terminus) and ran SDS-PAGE after transfection, in parallel with Flag-NS5B and Flag-NS5BΔC21aa. We found that the mobility on SDS-PAGE was quite comparable for all four proteins in three independent experiments (**Appendix Figure S3**), although the expression level of NS5B-Flag was at a lower level.

The TMD of transmembrane proteins is typically hydrophobic, while the extracellular structure is normally hydrophilic. In general, and in other experimental experiences, migration on SDS-PAGE that does not correlate with formula molecular weights appears to be common for membrane proteins. The anomalous gel shifting of NS5BΔC21aa may be due to the changes in its hydrophobicity, after removing TMD. The same phenomenon was also observed in previous reports, where the TMD of MAVS was removed, the mobility on SDS-PAGE appeared to be the same as that of full-length MAVS (Qin *et al*, 2017). Similarly, when we deleted the TMD of ATAD1, the migration of ΔTMD on SDS-PAGE appears similar to (or slightly slower) than that of full length ATAD1 (**Figure 4G**). We have added a comment/speculation to the anomalous migration of NS5BΔC21aa. Please see **Figure 5 legend**.

For more details on the gel shifting of membrane proteins, may be referenced to literatures (Cortas *et al*, 1991; Rath *et al*, 2009).



Appendix Figure S3. The mobility on SDS-PAGE of full length NS5B and NS5BΔC21aa.
293T cells were transfected with plasmids expressing NS5B and NS5BΔC21aa, with Flag-tag at the N-terminus (Flag-NS5B and Flag-NS5BΔC21aa) or at the C-terminus (NS5B-Flag and NS5BΔC21aa-Flag) for 24 hours, and then the cells were harvested and analyzed by western

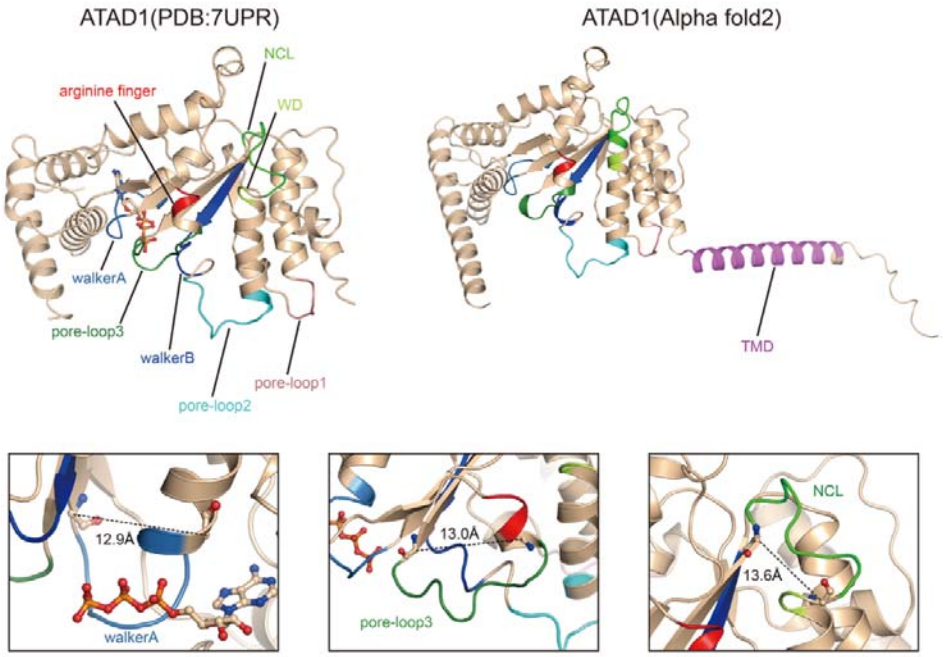
blotting with anti-Flag antibody.

Third, In Figure 4D, Zhou et al. use deletion mutants to examine the region of ATAD1 required for binding to NS5B, but I wondered if these deletion mutants of ATAD1 retain the tertiary and quaternary structures of native ATAD1. At the very least, by deleting the walker B, WD motif, and arginine finger of ATAD1, could it be that ATAD1 is destabilized?

Authors: We fully agree with this thoughtful comment. To explore the destabilization of nine deletion mutants of ATAD1, we performed the CHX chase experiment (new **Figure EV4A** and **4B**). We transfected 293T cells with HA-ATAD1 or ATAD1 domain-deletion mutants for 24 hours, and then the cells were treated with CHX (100 μg/mL) for indicated duration. The cells were harvested and analyzed by western blotting with anti-HA antibody. In addition, the ratio of HA:Tubulin were analyzed by gel band intensities using ImageJ software, with the relative amount of HA-ATAD1 or ATAD1 domain-deletion proteins at 0 hour set to 1. The data showed that mutants ΔTMD, Δpore-loop 1, and Δpore-loop 2 were still destabilized, but the stabilization of Δwalker A, Δwalker B, Δpore-loop 3, ΔWD motif, ΔNCL and Δarginine finger mutants was significantly reduced compared to the full length ATAD1. These data confirm that the lack of interaction between NS5B and ΔTMD, Δpore-loop 1, and Δpore-loop 2 was not attributed to potential destabilization of these mutants but rather due to undetectable interaction.

In addition, we also perform structural analysis and prediction of stability using existing ATAD1 structured (PDB: 7UPR) (Wang *et al*, 2022) and Alpha Fold2. These three mutants were most likely to maintain their stability (**Appendix Figure S5**).

The figure corresponding to this question have been revised, please see **Figures EV4A, 4B and Appendix Figure S5.**



Appendix Figure S5. The predicted structures of ATAD1 mutants.

According to the parsed ATAD1 (PDB: 7UPR) and the TMD structure model predicted by Alpha Fold2, there are flexible linkers with sufficient flexibility located at either end of pore-loop 1, pore-loop 2, and TMD domains. Upon deletion of pore-loop 1, pore-loop 2, or TMD domain, the linkers on both ends could come into contact without altering the secondary structure. However, in case of walker A, walker B, pore-loop 3, WD motif, NCL, or arginine finger domain, which have distant or stable secondary structures at their respective ends, deletion would result in a change in spatial location leading to instability.

Minor points

1. According to previous literature, NS5B is thought to have an RNA-dependent RNA polymerase domain at the N-terminus and a single TMD consisting of 21 amino acids at the C-terminus, taking the topology of a TA protein. However, the domain structure of NS5B and the putative location of the TMD are unclear in this manuscript. Please create a new figure to help the reader understanding.

Authors: We agree with this comment. Previous report demonstrated that the transmembrane proteins which can be delivered post-translationally to target in the membrane, including ER, outer mitochondrial membrane (OMM) or peroxisomes by a single TMD that no longer 30 amino acids in C-terminal are classified to the tail-anchored (TA) proteins (Borgese *et al*, 2003). To investigate the location of NS5B TMD, we generated the plasmid which containing a single TMD consisting of 21 amino acids at the C-terminus (21aa) of NS5B (pEGFP-C21aa) and the plasmid with a deletion of 21aa (pEGFP-NS5BΔC21aa). We co-transfected Huh7.5 cells with EGFP-NS5B, EGFP-NS5BΔC21aa or EGFP-C21aa with DsRed-Mito. We captured the images using Zeiss LSM800 (new **Figure 2B**) and analyzed their PCC by Imaris 8.4 software (new **Figure 2C**). The

absence of C21aa (pEGFP-NS5BΔC21aa) resulted in diffuse cytoplasmic signal and the bright spherical signals in nucleus, which was consistent with the previous report (Schmidt-Mende *et al*., 2001).

In addition, we can see that no significant difference was for the PCC between NS5B or C21aa, and mitochondria (PCC, 0.6339 and 0.5913), but the PCC of NS5BΔC21aa and mitochondria had virtually no co-localization (PCC, 0.1432). Taken together, our results demonstrated that the C21aa of NS5B determined its subcellular distribution, especially the localization with mitochondria.

The revised figure corresponding to this question are at Figure 2B and 2C.

Referee #2:

HCV infection is one of the leading reasons of CHC world widely. Protease and polymerase inhibitors could effectively cure HCV infection in clinics; however, we are still lack of understanding of virus-host interaction. In this manuscript, Li and his colleagues utilized HCV cell culture model in vitro to examine the ATAD1 on HCV infection. They found that ATAD1 KO could dramatically enhance HCV infection, and mechanistic studies found that ATAD1 could interact with viral NS5B protein and then cause NS5B degradation via proteasome pathway. Overall, this manuscript is well organized and the experiments were well controlled. I have three major questions for the authors to be addressed:

Authors: We thank this reviewer for your positive comments to our study. We have revised the manuscript by following your comments below.

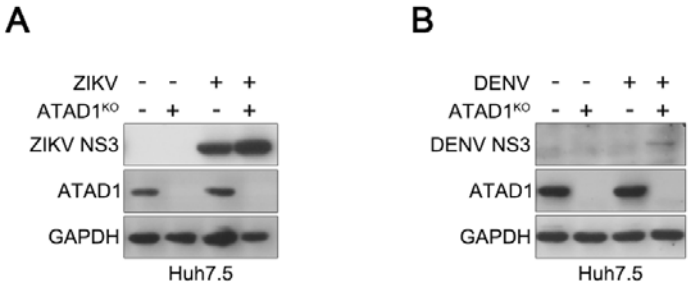
1. The authors should exclude other reasons which lead to enhanced HCV infection when ATAD1 KO. As ATAD1 is responsible for mitochondrial homeostasis, and MAVS localized at mitochondria, is it possible that ATAD1 KO could impair the antiviral immunity leading to enhanced HCV infection?

Authors: We appreciate those interesting comments and constructive suggestions. To address this, we have performed additional experiments and generated significant amount of data, as shown in new **Figure 6**. We generated MAVS knockout cells in WT and ATAD1^{KO} Huh7.5 cells, named MAVS^{KO} and MAVS^{KO}/ATAD1^{KO} cells. We infected WT, ATAD1^{KO}, MAVS^{KO} and MAVS^{KO}/ATAD1^{KO} cells with JFH1. The total level of MAVS expression was not significantly affected by ATAD1 knockout. However, extensive cleavage of MAVS was observed upon HCV infection, accompanied with a decreased TBK1 activation and the prion-like aggregates of MAVS in ATAD1^{KO} Huh7.5 cells. Nevertheless, ATAD1 remained a negative regulator for HCV infection even in the absence of MAVS, demonstrated an increased HCV infection in MAVS^{KO}/ATAD1^{KO} cell compared to that in ATAD1^{KO} Huh7.5 cells. Thus, we conclude that ATAD1 could also augment the antiviral function of MAVS, but this effect was independent of its action on NS5B.
The underlying mechanism of ATAD1-MAVS interaction warrants additional investigations.
We have added new data in a new section, accompanying with Figure 6. Please see **Figure 6.**

2. According to the mechanism of ATAD1 in this study, the reviewer is curious whether ATAD1 function is conserved in other RNA viruses or even DNA viruses? The authors could use other RNA viruses, such as ZIKV or DENV to test the specificity.

Authors: We thank the reviewer for this suggestion. By following your suggestion, we had performed ZIKV and DENV infection experiments in the WT and ATAD1^{KO} Huh7.5 cells, and we found that knockout of ATAD1 slightly enhanced ZIKV and DENV infections in two independent experiments. However, by considering the focus and logic flow of this study, plus the time limit for an in-depth elucidation of the regulatory role of ATAD1 on ZIKV and DENV infection, we tended not to include these data into the manuscript. For the same considerations, the role of

ATAD1 on other DNA virus, e.g. HSV-1, could be tested in the future.



Appendix Figure S1. Knockout of ATAD1 slightly enhanced ZIKV and DENV infections in Huh7.5 cells.

(A and **B)** WT and ATAD1^{KO} Huh7.5 cells were infected with ZIKV (A) or DENV (B) for 36 hours, and then the cells were harvested and analyzed by western blotting with anti-NS3 and anti-ATAD1 antibodies.

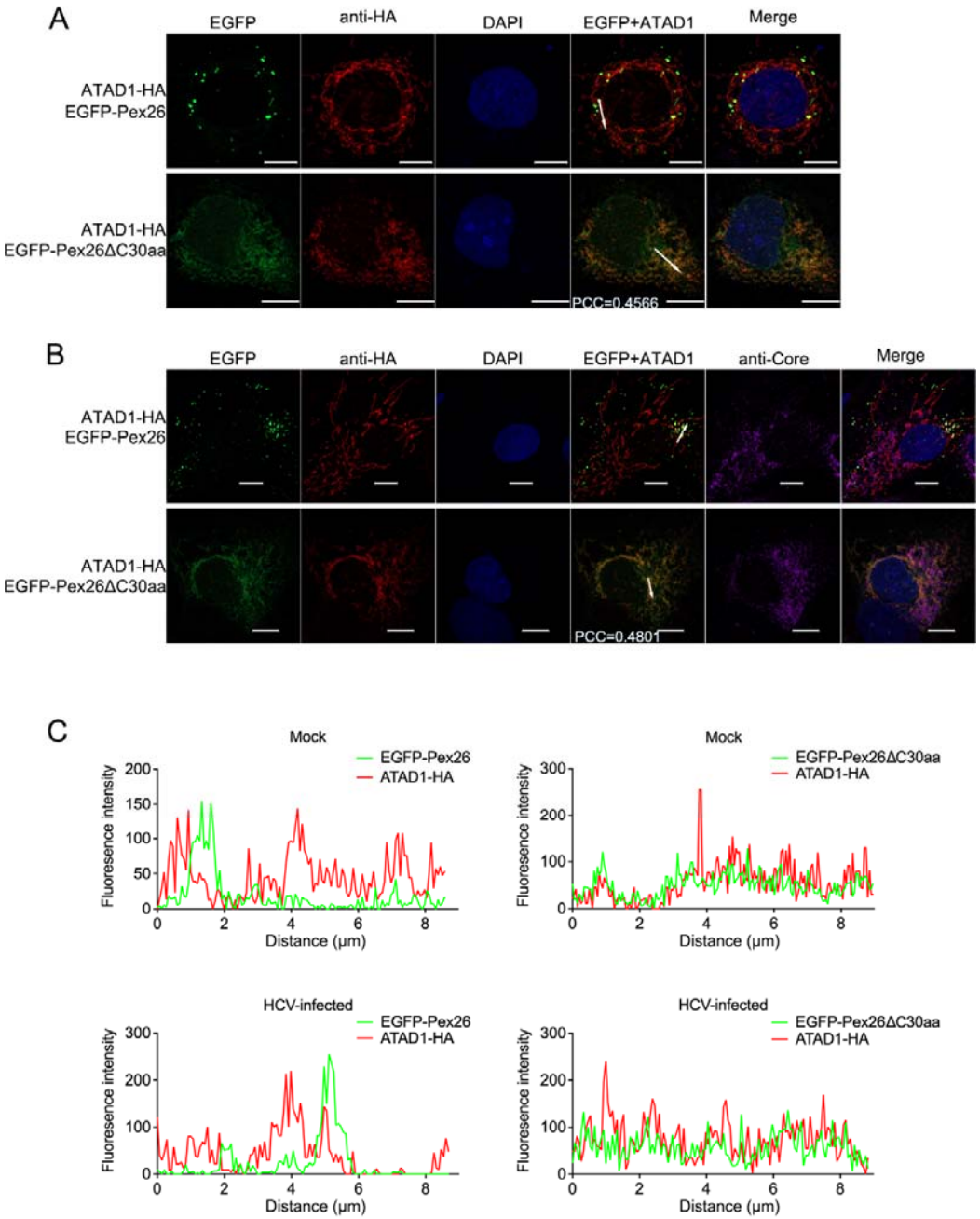
3. the authors could further clarify the rationales to test ATAD1 on HCV infection. is there any relevance of ATAD1 in HCV pathogenesis? Is there any evidence that HCV infection impair ATAD1 function at mitochondria?

Authors: We appreciate this comment. We have added the rationales to test ATAD1 on HCV infection. Please see the manuscript.

This is no previous report on the correlation between HCV and ATAD1. In our study here, we performed HCV infection in vitro, and the results obviously demonstrated that HCV NS5B mislocalized to mitochondria and caused mitochondrial fragmentation, while ATAD1 was responsible for removing the mislocalized NS5B from the mitochondria, thus maintaining a normal mitochondria homeostasis. While it is well known that a quality-controlled mitochondria is vitally important for the cellular functions, and a dysfunction of mitochondria is associated with a range of diseases, including cancer. However, given these in vitro data only, we are hesitating to speculate the relevance of ATAD1 in HCV pathogenesis. However, given our findings that ATAD1 exerts an antiviral effect against HCV infection, we are interested in exploring whether there is any relevance in HCV pathogenesis by analyzing clinical samples in the future.

In this study, we primarily focused on the impact of ATAD1 on viral NS5B and its subsequent effect on HCV infection. We are also interested in whether HCV infection affected in turn the function of ATAD1 on mitochondria. However, due to the technical issues, we were unable to design an experiment that could optimally reveal the function of ATAD1 upon HCV infection. However, we had tried to design an experiment to examine the function of ATAD1 on mitochondria. Specifically, it has been reported that ATAD1 physically interacts with mislocalized TA protein Pex15, and its homologous protein in mammals is Pex26. Pex26 is typically targeted to peroxisomes, but Pex26ΔC30aa will be mislocalized to mitochondria and interact with ATAD1. We generated the plasmids expressing EGFP-Pex26ΔC30aa and the full length EGFP-Pex26. We

co-transfected Huh7.5 cells with EGFP-Pex26 or EGFP-Pex26ΔC30aa with ATAD1-HA for 24 hours (**Appendix Figure S4A**). Meanwhile, we infected Huh7.5 cells with JFH1 for 24 hours, and then co-transfected with EGFP-Pex26 or EGFP-Pex26ΔC30aa with ATAD1-HA for 24 hours (**Appendix Figure S4B**). We examined the interaction between ATAD1 and EGFP-Pex26ΔC30aa by confocal microscopy. The results revealed that EGFP-Pex26ΔC30aa but not EGFP-Pex26 co-localized with ATAD1 to a comparable extent, with or without HCV infection (**Appendix Figure S4**). These data demonstrated that HCV infection did not apparently affect the function of ATAD1 in terms of its interaction with the TA proteins. Therefore, we believe that, as a quality control of mitochondria, ATAD1 was not apt to be disrupted upon HCV infection. However, this is not in the streamline of this study, and we did not investigate further for its endogenous interaction with Pex26ΔC30aa, because the production of anti-ATAD1 antibody we used previously has been halted (Abcam, ab94583).



Appendix Figure S4. HCV infection did not apparently affect the function of ATAD1 in terms of its interaction with the mitochondria-targeting TA-protein Pex26 mutant.

(A) Huh7.5 cells were co-transfected with EGFP-Pex26 or EGFP- Pex26ΔC30aa with ATAD1-HA for 24 hours. The cells were fixed and blotted with rabbit primary antibody anti-HA for 2 hours at room temperature, followed by incubation with Goat anti-Rabbit conjugated IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 647 conjugate for 1 hour at room temperature. Immunostaining of ATAD1 was in red color, while nuclei were stained with Hoechst in blue color. Pex26 and Pex26ΔC30aa were visualized by EGFP (green). The PCC between Pex26ΔC30aa and

ATAD1 were shown in the image using white color. The direction of arrow corresponds to the horizontal coordinate in ***panel C***. Scale bars, 10 μm.

(**B**) Huh7.5 cells were infected with JFH1 for 24 hours, and then co-transfected with EGFP-Pex26 or EGFP- Pex26ΔC30aa with ATAD1-HA for 24 hours. The cells were fixed and blotted with rabbit primary antibody anti-HA (red) and mouse primary antibody anti-Core (purple) for 2 hours at room temperature. Secondary antibody Goat anti-Rabbit conjugated IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 647 conjugate (HA, red) and Goat anti-Mouse conjugated IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor® 568 conjugate (Core, purple) were incubated for 1 hour at room temperature. Nuclei were stained with Hoechst (blue). Pex26 and Pex26ΔC30aa were visualized by EGFP (green). The PCC between Pex26ΔC30aa and ATAD1 were shown in the image using white color. The direction of arrow corresponds to the horizontal coordinate in ***panel C***. Scale bars, 10 μm.

(**C**) Distant colocalization analysis of Pex26 or Pex26ΔC30aa and ATAD1 in ***panels A*** and ***B*** were performed using Zen and processed by GraphPad Prism.

Referee #3:

Zhou et al identify the mitochondrial protein ATAD1 as a negative regulator of hepatitis C virus infection. The authors employ a variety of techniques (CRISPR/Cas9; high resolution microscopy) and different HCV isolates to examine how ATAD1 influences HCV replication. In particular, they find that ATAD1 removes and directs the HCV protein NS5A towards proteasomal degradation. Despite decades of research the live cycle of HCV and the role of various host factors remains incompletely understood. This study is therefore another piece in the puzzle. Overall the content of the manuscript is well structured and the experiments are performed at a very high quality (in particular the western blots). The authors might consider to address the following minor points to further improve the quality of their manuscript:

Authors: We thank this reviewer for your positive comment for our study. By following your comments and suggestions, we have performed additional experiments to address the questions below and revised the manuscript accordingly.

Major comments:

The authors mainly use core protein western blots as a readout for HCV infection and only provide infectious titers for a single HCV isolate (JFH1). They might consider to confirm increase of infectious viral titers upon ATAD1 knockout for further HCV isolates.

Authors: Thank you for this comment. By following your suggestions, we have tested the infections of other HCV genotypes in WT, ATAD1^{KO} and ATAD1^{KO+Res} Huh7.5 cells and determined the viral titers of them by FFU assay using anti-Core (new **Figure EV2D**) and western blotting for Core and NS5B proteins (new **Figure EV2E**). This has included the viral titers of HCV TNcc (genotype 1a), JFH1 (2a), J6cc (2a), DBN (3a), ED43$^{5'UTR\text{-}NS5A}$/JFH1 (4a/2a chimera), SA13$^{5'UTR\text{-}NS5A}$/JFH1 (5a/2a), HK6a$^{5'UTR\text{-}NS5A}$/JFH1(6a/2a). The results demonstrated that knockout of ATAD1 enhanced the infection of different HCV genotypes, either full-length clones or chimera.

The figure corresponding to this question are at Figure EV2D and 2E.

Moreover, the authors could distinguish whether ATAD1 knockdown leads to an increased number of infected cells or the same number of infected cells but with higher levels of the core protein (i.e. via immunofluorescence of the core protein).

Authors: Thank you for this interesting question. In Figure **EV2B**, we have demonstrated that knockout of ATAD1 leads to an increased number of J6/JFH1-EGFPΔ40-infected cells, indicating by the Core positive cell numbers.

In addition, by following your suggestion, we performed additional experiments and analyzed the amount of HCV proteins using flow cytometry for detecting median fluorescence intensity (MFI) of NS5A-EGFP fusion protein in J6/JFH1-EGFPΔ40-infected cells (new **Figure 1O-P**). Briefly,

WT, ATAD1^{KO} and ATAD1^{KO+Res} Huh7.5 cells were infected with J6/JFH1-EGFPΔ40 [namely 2a(J6)-EGFPΔ40 in (Gottwein *et al*, 2011)] for 72 hours. The percentage of GFP-positive cells (new **Figure 1O**) and MFI (new **Figure 1O**) in HCV infected cells were significantly increased in ATAD1 knockout cells, which could be suppressed down by the complementation of ATAD1 (three independent experiments were shown in different colors). Data are represented as mean ± SEM. One-way ANOVA with Dunnett's post-hoc test was used to analyze the difference (comparing to ATAD1^{KO} group). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

The immunofluorescence (**Figure EV2B**) and flow cytometry analysis (new **Figure 1O-P**) demonstrated that ATAD1 knockout led to an increased number of HCV infected cells and a higher level of HCV infection in each infected cell.

The revisions corresponding to this question are shown in Figure 1O-P and theirs legends.

The authors may determine colocalization of NS5B and Mito in HCV-infected cells. The analysis of NS5B localization is mainly based on vector-based expression of NS5B.

Authors: We appreciate this insightful comment and we are also very interested in knowing the localization of infection-produced viral NS5B. Actually, we have performed experiment to look at the localization of virus-expressed NS5B previously, however the NS5B antibody did not work for immunofluorescence analysis, but work in western blotting.

By following your suggestion, we have been trying to perform the experiment to determine the co-localization of NS5B and mitochondria again, using other antibodies commercially available. We tested NS5B antibodies that were previously used in other studies, however it appeared to stain the whole cell contents without showing NS5B-specific signal (Zhao *et al*, 2017). Then, we tried another NS5B antibody as previously used (Manabe *et al*, 1994), again we were unable to obtain specific fluorescence signal. Then, we tested Abcam (ab122972) and Gentex (GTX00713), but both did not work.

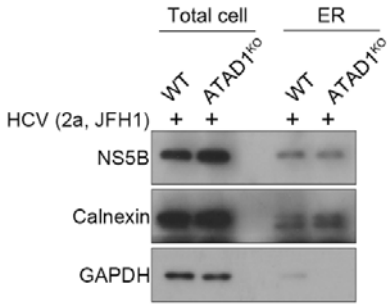
In addition, we have attempted to construct an NS5B-tagged HCV recombinant for visualization of NS5B during infection; however insertion of a Flag tag or EGFP were deleterious. The NS5B-tag recombinant was nonviable after transfection.

Nevertheless, we have included other experiments to reveal the co-localization/association of NS5B and mitochondria (**Figure 5E**). We isolated mitochondria from cytoplasm contents following HCV infection, and demonstrated that a fraction of NS5B co-localized with mitochondria (**Figure 5E**). We also observed the presence of NS5B in the cytoplasmic contents (**middle, Figure 5E**).

Furthermore, we also performed additional experiment to determine the co-localization of NS5B and ER **(Appendix Figure S2)**. We infected WT and ATAD1^{KO} Huh7.5 cells with JFH1 for 72

hours. The cells (5 × 10^7 cells) were harvested and used for ER isolation by Minute^{TM} ER enrichment Kit (Invent Biotechnologies, ER-036, USA). Equal amounts of protein (2.7 μg) were analyzed by western blotting with anti-NS5B and anti-Calnexin antibodies. Calnexin was detected as the ER loading control, and GAPDH was detected as the total cell control. The results demonstrated that NS5B had co-localization with ER in HCV-infected cells. Noting that the amount of ER-associated NS5B was similar in both WT and ATAD1^{KO} Huh7.5 cells, which is in consistence with the analysis of co-localization by confocal microscopy **(the PCCs, Figure 3A and C)**. Taken together, these isolation experiments of subcellular contents demonstrate that NS5B was co-localized with the mitochondria and ER in HCV-infected cells.

The revisions corresponding to this question are shown in Appendix Figure S2.



Appendix Figure S2. The co-localization of NS5B with ER in HCV-infected cells.
WT and ATAD1^{KO} cells were infected with JFH1 for 72 hours, and then the cells were harvested and isolated for ER using the Minute^{TM} ER enrichment Kit (Invent Biotechnologies, ER-036). Equal amounts of protein (2.7 μg) were analyzed by western blotting with anti-NS5B and anti-Calnexin antibodies.

There is a discrepancy of the PCC in different Figures, although the experiment is the same. The PCC of NS5B and Dsred-Mito in Figure 2C is 0,75 and in Figures 3c it is 0,5.

Authors: Thank you for the insightful comments. We have looked into the analysis for images in original Figure 2C and 3C and identified the analysis performed for original Figure 2C was done by IPP software, while other PCC analyses were done by Imaris. The algorithm in two software may be different, thus the PCCs showed differences.

While to determine the co-localization of NS5B and ER in ATAD1 knockout cells (Figure 3A), we have performed new experiment to generate new images with higher quality. These allowed us to capture more confocal images with higher quality for NS5B and DsRed-Mito as well and analyze all the images with identical procedures. Therefore, the original version of images has been replaced with new images **(new Figure 2B-C and Figure 3A-B)**, with new PCCs from the analysis of co-localization for NS5B and DsRed-Mito.

The revisions corresponding to this question are shown in Figure 2B-C and Figure 3A-B.

Minor comments:

Figure 2A: The fragmentation of the mitochondria is hard to judge from a single image of one cell, the authors might explain more thoroughly in the text how they define and quantify mitochondria fragmentation.

Authors: By following your suggestion, we have added the details by which we defined the fragmentation and linear for mitochondria to the text. Following acquisition of high-quality confocal images, we utilized ImageJ software to determine the mean length of mitochondria, with a threshold of 2 μm used to distinguish between linear and fragmented forms.

We have added the description in Materials and Methods.

Figure 2C: The coloring of the median line/error bars or symbols might be adapted (now its black on black).

Authors: Thank you for this good suggestion. We have changed the line/error bars and symbols into the different color in the figures.

Figure 3B: The panels for WT and ATAD1-KO seem mislabeled, given that for the KO cells a stronger co-localization is expected as seen in 3C.

Authors: Thank you for pick out this mistake. We have replaced the old Figure 3 with the **new Figure 3**, taken from new experiments.

Only Student's unpaired t-test was performed, this does not seem appropriate for all data sets here, since sometimes multiple comparisons were performed.

Authors: Thank you very much for point this out. We have re-analyzed the data in Figure 1N, 1O, 1P and Figure EV 2A-D using one-way ANOVA with Dunnett's post-hoc test, Figure 2E and EV3B using two-way ANOVA with Tukey's post-hoc test.

Line 382. The authors wrote that they "analyzed the co-localization by confocal microscopy" but only show a single image for each condition. Please provide more comprehensive analysis or rephrase.

Authors: Thank you for your suggestions. We have incorporated the Pearson correlation coefficient (PCC) analysis to evaluate the co-localization of ATAD1 with EGFP-NS5B, as shown in **Figure 4A** and **4B**. It is worth noting that PCC was also utilized in other relevant co-localization analysis.

Please proofread the whole manuscript. There are several grammar errors and typos (i.e. Fig S1E "fag" instead of "flag").

Authors: We have corrected these errors (e.g. Figure EV1E). Meanwhile, we have thoroughly proofread the entire manuscript.

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Dear Prof. Li

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript, but we have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and support publication.

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.

- Please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- Please remove the Author Contributions from the manuscript file and make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish.

- Please note that all data mentioned/described in the manuscript must be available to the reader and shown. Please apply this to the "data not shown" statement on page 29.

- Please add callouts in the text to the Appendix Figures and their panels.

- Appendix: please add a title page and table of content with page numbers.

- Please remove the list of abbreviations from the manuscript file and make sure to define all abbreviations in the manuscript text when they first appear.

- Please order the manuscript sections as follows:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please correct the heading "Supplementary Figures" to 'Expanded View Figure Legends'.

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Referee #3:

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Prof. Yi-Ping Li
Sun Yat-sen University
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Zhongshan II Road
Guangzhou, Guangdong 510080
China

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The data shown in figures should satisfy the following conditions:

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