VPS13D interacts with VCP/p97 and negatively regulates ER- mitochondrial interactions

Yuanjiao Du, Jingru Wang, Juan Xiong, Na Fang, and Wei-ke Ji

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Ji:

Thank you very much for submitting your paper entitled "VPS13D couples with VCP/p97 to safeguard ER-mitochondria interactions" to MBoC. Two referees have seen your work. Both felt that your findings, in principle, would be of interest to our readership. Please find their reports attached below. They recommend, among a few other points, to perform a set of control experiments to further substantiate your model.

In sum, we would be happy to consider a revised manuscript that satisfies the joint concerns of the referees. Normally, if additional experiments are needed a paper is rejected. However, these experiments can be performed relatively easily and follow directly from your current results.

Therefore, we look forward to receiving your revised manuscript, together with a letter indicating the changes you have made and your responses to the referees.

Sincerely,

Martin Ott
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Ji,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The manuscript entitled "VPS13D couples VCP/p97 to safeguard ER-mitochondria interactions" by Du et al. studies the interplay between VPS13D and p97 in mammalian cell lines. Strikingly, the authors show that knockdown of VPS13D leads to hyper-tethering of the ER to the mitochondrial network, leading to a wide range of perturbations in mitochondrial homeostasis. They propose that the large upregulation of ER-mitochondria contact sites observed is a consequence of a downregulation of p97 in their VSP13D knock down conditions. To reach this conclusion, they provide some causal evidence that affecting p97 phenocopies VPS13D mutation. They also propose that p97 levels are directly regulated by interaction with VPS13D, and that p97 loss affects VAPB levels/activity, leading to increased tethering. The evidence for these two ideas is more circumstantial. Therefore, how VPS13D inactivation leads to ER-mitochondria hypertethering remains largely unexplored.

The findings - in particular, that loss of VPS13D leads to hyper-tethering of the ER and mitochondria and a dramatic reduction in p97 levels - are interesting and generally well controlled. There are, however, some considerations before publication:

1. Figure 1F shows a clear mitochondrial localization for VPS13D. The quantification of the data in Figure 1G&H, however, does not rule show the proportion of a random mitochondrial protein at ER-mitochondria junction. Because the ER covers a large proportion of the cell, any mitochondrial protein will be found at ER-mitochondria junction by chance. What is the chance to find any random bit of mitochondrion in contact with the ER, and is it different from the proportions reported for VPS13D?

2. Similarly, the authors conclude that VAPB is enriched at ER-mitochondria contact sites in the VPS13D knockdown conditions. But since the whole ER is hypertethered in these conditions, it is difficult to decide whether this enrichment is specific for VAPB, or whether it would be observed for
any general ER protein. This should be relatively simple to test with comparisons to other ER markers.

3. There are currently a couple of issues with the data presented in Figure 3B. Firstly, there are no conditions that show parts of VPS13SD that do not interact with p97, i.e. a VPS13D negative control. The only negative control herein is GFP alone. However, if, for instance, attachment of a protein fragment would cause misfolding, the GFP-trap might be detecting aggregation rather than specific interaction. Secondly, there isn't any UBA-GFP on the presented blots. Indeed, it is difficult to know what size this fragment should be, as Figure 3A currently states that both the UBA and VAB fragment finish at amino acid 3549 (likely a mistake).

4. The use of a p97 allosteric inhibitor makes a good case in support of the model that the hyper-tethering observed upon VPS13D knockdown is caused to decreased p97 activity downstream, thus providing a solid causal link. The causal link is important and would be much better supported by the inclusion of both p97 knockdown (to ward off any non-specific activity of the drug) and overexpression (to show if restoring p97 to its normal level in VPS13D deficient cells restores the phenotype).

5. Figure 3C shows that VAPB levels decrease when p97 is overexpressed. This figure also attempts to show that pathogenic VAPB mutants are refractory to p97-mediated downregulation. However, the figure does not include any control conditions for the mutant constructs, i.e. what are the levels of the mutant construct in the absence of p97 overexpression? Could it be that VAPB-T46I is still sensitive to p97-mediated repression, but starts from a much higher level than the WT in the absence of p97 overexpression?

6. The apparent reduction in the interaction between p97 and VAPB appears simply to be a result of decreased p97 levels (Fig 3I). This interpretation should be considered.

7. As said above, it is not clear that any of the phenotypes observed are a consequence of VPS13D and p97 interacting. The role of p97-driven downregulation of VAPB is also superficially tested. For instance, it is unlikely that the 2-3x overexpression of VAPB observed in upon VPS13D knockdown (Fig 3G) is solely responsible for the hypertethering observed here. This is unlike p97 inactivation, which appears to be sufficient to explain hypertethering (Fig 3 L-M). This should be at thoroughly acknowledged in the text.

Minor comments
1. On page 4 the authors state "All of these ER-associated MCSs were not substantially altered upon VPS13D suppression". The language here is quite strong. It might be more appropriate to state that the authors did not detect any alterations in ER-associated MCSs.
2. At the end of the first paragraph on page 5 (regarding figure S3) the authors could also conclude that the hyper-tethering does not require the presence of VPS13A as they still see hyper-tethering in the VPS13A/D double knockdown condition.
3. Figure S4 includes panel I which is a replica of Figure 2D. Probably a mistake since S4I is not referenced in the text.
4. The authors show convincing double knockdown of VAPB and PTPIP51 in Figure 2D; however, it would also be important to show that VAPB and PTPIP51 are still as efficiently reduced in the VPS13D, VAPB and PTPIP51 triple knockdown cells as in the single KD.
5. Page 9: "...VPS13D suppression strongly diminished the localization of p97 puncta at ER-mitochondria MCSs". From the data presented, it is difficult to know whether p97 is specifically diminished from ER-mitochondria MCSs, as opposed to the levels simply being reduced everywhere in the cell (fitting with the data in Figure 3G).
6. The error bars in Figure 4C seem non-sensical given that it is a binary phenotype, i.e. cells either do, or do not, exhibit a perturbed mitochondrial morphology. The proper statistics to use in binary data and contingency tables are for instance the chi-squared or Fisher exact tests.
7. The calcium data are interesting but hard to interpret. First, experiments are performed in Opti-MEM, the composition of which is unknown to this reviewer. Most important is the calcium content.
of this medium. If high (mM range), then ionomycin most likely causes mitochondrial calcium intake independent of the ER, by permeabilization of the plasma membrane. Either a calcium-depleted medium or a physiological stimulation of IP3 receptor should be used to show increased ER-mitochondria calcium exchange. Second, it isn’t clear at which level the mitochondrial calcium marker saturates. It is quite likely that the absence of large difference in the peak mitochondrial calcium levels are due to saturation of the biosensor.

8. Fig. 2A shows a rescue of hypertethering by knockdown of VAPB and PTPIP51. It would be interesting to know whether the defects in mitochondrial morphology (Fig 4B-C) are rescued as well. Presumably, this should be easy to quantify from the images the authors already have.

9. Having a third of the discussion on the link to lipid droplets seems peculiar when so little of the work presented focuses on lipid-droplets.

Reviewer #2 (Remarks to the Author):

In their manuscript "VPS13D couples with VCP/p97 to safeguard ER-mitochondria interactions", the authors show that VPS13D controls the extend of contact established between the ER and mitochondria, most probably via altering the levels of the tether VAPB via decreased degradation mediated by p97. Hypertethering induced by depletion of VPS13D caused a substantial enwrapping of mitochondria in ER tubules and affected mitochondrial morphology and distribution, leading to a rather fragmented instead of elongated mitochondrial network. In addition, the authors report on some interesting implications of VPS13D-induced hypertethering for mtDNA replication and mitochondrial calcium handling.

This study is well-done and data and figures are of high quality. The manuscript is well written, and the conclusions drawn by the authors are sound and well justified by presented data and appropriate controls. While a plethora of different tethering components between the ER and mitochondria have been identified in the last years, it is still not clear how these molecular tethers are controlled. This study provides novel insights into the control of ER-mitochondria contact by VPS13D/VAPB/p97, and though the precise molecular events involved are not completely clear, these findings will be interesting for the field.

I only have a few comments and suggestions:

1. The observation that ER-mitochondria hypertethering induced by depletion of VPS13D is accompanied by a quite dramatic drop in p97 and depletion or inhibition of p97 itself causes hypertethering as well is highly interesting. The authors suggest that this decreased abundance of p97 and subsequent increased levels of the tether VABP due to lack of proteolytic removal might be the reason for VPS13D-induced hypertethering. While it is already shown that increased levels of VAPB increase ER-mitochondria contact area, it is novel that this can be regulated by VPS13D, potentially via p97-mediated degradation. The hypothesis of the authors that VPS13D affects VAPB levels via p97 (as also indicated in the title) should be tested to establish causality between observed effects.

   e.g. via testing whether VPS13D depletion still causes hypertethering when p97 levels are high, e.g. via overexpression? Can some of the phenotypes in respect to ER-mitochondria contact observed upon VPS13D depletion be prevented when p97 levels do not decrease?
2. How could VPS13D depletion lead to this drastic drop in p97 levels? Experiments to address this might be beyond the scope of this study, but could the authors provide a section in the discussion to speculate on possible underlying reasons for the strong decrease of p97 when they deplete VPS13D?

3. VPS13D silencing seems to not only result in increased contact area between ER and mitochondria but to also cause a prominent change in mitochondrial network morphology, leading to a strong fragmentation of mitochondria (Fig. 4). Earlier in the manuscript, the authors show that when they deplete VAPB and PTPIP51, they can prevent hypertethering induced by VPS13D depletion. According to the microscopy pictures shown in Fig. 2F, this also seems to restore an elongated mitochondrial network, though this is hard to judge and the authors did not comment on it. Maybe the authors could consider to additionally quantify mitochondrial perimeter from those pictures, just as they have done for VPS13D depletion alone in Fig. 4. This might show that the alterations in mitochondrial morphology and distribution caused by VPS13D depletion are indeed caused by VPS13D-VAPB mediated hypertethering (as hypothesized by the authors in the discussion).

4. The authors use crude mitochondrial fractions to show that VAPB is more abundant at MAMs/mitochondria when VPS13D is missing. This looks convincing, but it would be good to discriminate between a general increase in VAPB protein levels in total cell lysates and a selective increase of VAPB at the MAMs/mitochondria to see whether VAPB is indeed more efficiently recruited to the contact sites. Does the increase of VABP at MAMs/mitochondria only mirror the increase of VAPB in general (as for instance shown in Fig 3G at the total cellular level) or is it also more prominently recruited to MAMs?

Minor:

Mostly, statistics seem correct, but for instance in Fig. 2E or S4H, an unpaired student t test was used. However, these are multiple comparisons and thus require the use of a one-way ANOVA to compare between all groups.

The same immunoblots to detect VAPB and PTPIP51 are shown twice (Fig. 2D and Fig. S4I)

Is the coimmunoprecipitation in Fig. 3B lacking a panel to detect UBA-GFP?

Fig. 1A shows a very stunning effect of VPS13D silencing on ER-mitochondria contact area in U2OS cells, and the authors refer to Fig. S1A and S1B for respective controls of efficient depletion. However, this now shows an immunoblot for VPS13D depletion in HEK293 cells (S1A) and does not state information about which cell line was used for the RT-qPCR conformation of efficient silencing (Fig. S1B). This needs to be clarified and corresponding controls for the data shown in Fig.1 should be included.
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1. Figure 1F shows a clear mitochondrial localization for VPS13D. The quantification of the data in Figure 1G&H, however, does not rule show the proportion of a random mitochondrial protein at ER-mitochondria junction. Because the ER covers a large proportion of the cell, any mitochondrial protein will be found at ER-mitochondria junction by chance. What is the chance to find any random bit of mitochondrion in contact with the ER, and is it different from the proportions reported for VPS13D?

We thank reviewer for this insightful comment. As shown in a recent study (Abrisch et al. 2020 Journal of Cell Biology), we set up a negative control, in which the VPS13D image is rotated 90° relative to the ER image, and we quantify the percentage of VPS13D puncta or foci at the ER contacts. We found that the percentage of VPS13D at ER contacts is substantially reduced (Fig. S3E), suggesting that the presence of VPS13D at ER contacts is significant and not due to chance. Since this result was reported in a very recent paper (Guillén-Samander et al., 2021), we referred to this recent work and moved these figures to the Fig. S3D, E, & F in the revised manuscript.
2. Similarly, the authors conclude that VAPB is enriched at ER-mitochondria contact sites in the VPS13D knockdown conditions. But since the whole ER is hypertethered in these conditions, it is difficult to decide whether this enrichment is specific for VAPB, or whether it would be observed for any general ER protein. This should be relatively simple to test with comparisons to other ER markers.

We thank reviewer again for this insightful comment. To answer this question, we examined the distribution of sec61, a general ER membrane marker, in cells depleted of VPS13D. VAPB was enriched at mitochondrial regions, likely representing ER-mitochondrial contacts in VPS13D-suppressed cells (Fig. 2A, middle panel). In contrast, the sec61 protein is almost evenly distributed all over the ER though we can still see enhanced ER-mitochondrial interactions in VPS13D-suppressed U2OS cells (Fig. 2A, bottom panel). This result suggests that the enrichment of VAPB at ER contacts is specific upon VPS13D suppression.
3. There are currently a couple of issues with the data presented in Figure 3B. Firstly, there are no conditions that show parts of VPS13SD that do not interact with p97, i.e. a VPS13D negative control. The only negative control herein is GFP alone. However, if, for instance, attachment of a protein fragment would cause misfolding, the GFP-trap might be detecting aggregation rather than specific interaction. Secondly, there isn’t any UBA-GFP on the presented blots. Indeed, it is difficult to know what size this fragment should be, as Figure 3A currently states that both the UBA and VAB fragment finish at amino acid 3549 (likely a mistake).

1) Firstly, there are no conditions that show parts of VPS13SD that do not interact with p97, i.e. a VPS13D negative control. The only negative control herein is GFP alone. However, if, for instance, attachment of a protein fragment would cause misfolding, the GFP-trap might be detecting aggregation rather than specific interaction.

Thanks for the comment. As reviewer suggests, we set up a VPS13D negative control (the C-terminal region of VPS13D (residues 3791-4363)) in a GFP-trap assay. We found
that the C-terminal region of VPS13D, named VPS13-C, fail to interact with p97, supporting that VPS13D-p97 interaction is specific.

2) Secondly, there isn’t any UBA-GFP on the presented blots. Indeed, it is difficult to know what size this fragment should be, as Figure 3A currently states that both the UBA and VAB fragment finish at amino acid 3549 (likely a mistake).

We thank reviewer for catching the typo. We correct the typo in Fig. 3A and the modified blots are shown in Fig. 3B. In addition, we provided an un-cropped blot for all the GFP-tagged VPS13D fragments.

4. The use of a p97 allosteric inhibitor makes a good case in support of the model that the hyper-tethering observed upon VPS13D knockdown is caused to decreased p97 activity downstream, thus providing a solid causal link. The causal link is important and would be much better supported by the inclusion of both p97 knockdown (to ward off any non-specific activity of the drug) and overexpression (to show if restoring p97 to its normal level in VPS13D deficient cells restores the phenotype).
We thank reviewer for this insightful suggestion. We examined the ER-mitochondria interactions in p97 siRNA treated U2OS cells. p97 is knocked down by two siRNA duplexes, and each of them works well (Fig. 3M). We found that p97 suppression mediated significantly enhances the ER-mitochondrial interactions (Fig. 3L and 3N), consistent with the p97 inhibitor results. In addition, we examined the ER-mitochondrial interactions in response to p97 overexpression in VPS13D-suppressed U2OS cells. Cells with VPS13D suppression and GFP vector expression substantially increases the ER-mitochondrial connections. Expression of p97-GFP moderately decrease, but not completely rescue, the ER-mitochondrial interaction at both peri-nuclear and periphery regions in VPS13D-suppressed cells (Fig. 3L and 3N), suggesting that, in addition to the level of p97, unknown pathways may also be responsible for the ER-mitochondrial hypertethering phenotype observed in VPS13D-suppressed cells.
5. Figure 3C shows that VAPB levels decrease when p97 is overexpressed. This figure also attempts to show that pathogenic VAPB mutants are refractory to p97-mediated downregulation. However, the figure does not include any control conditions for the mutant constructs, i.e. what are the levels of the mutant construct in the absence of p97 overexpression? Could it be that VAPB-T46I is still sensitive to p97-mediated repression, but starts from a much higher level than the WT in the absence of p97 overexpression?

Thanks for the insightful comment. We examined the level of VAPB and its pathogenic mutants in the absence of p97 overexpression in U2OS cells. We found that there is no substantial difference in the level of VAPB-GFP, VAPB-GFP-T46I, and VAPB-GFP-P56S (Fig. S5H).

6. The apparent reduction in the interaction between p97 and VAPB appears simply to be a result of decreased p97 levels (Fig 3I). This interpretation should be considered.

We thank reviewer for this useful comment. We discuss this interpretation in the result section of the revised manuscript.

7. As said above, it is not clear that any of the phenotypes observed are a consequence of VPS13D and p97 interacting. The role of p97-driven downregulation of VAPB is also superficially tested. For instance, it is unlikely that the 2-3x overexpression of VAPB observed in upon VPS13D knockdown (Fig 3G) is solely responsible for the hypertethering observed here. This is unlike p97 inactivation, which appears to be sufficient to explain hypertethering (Fig 3 L-M). This should be at thoroughly acknowledged in the text.

We thank reviewer for this great comment. We agree with reviewer that the overexpressed VAPB resulted from VPS13D suppression correlates with but may not be 100% responsible for such a strong tethering between ER and mitochondria. We think that multiple pathways may be responsible for the ER-mitochondrial hyper-tethering in
VPS13D suppressed cells. Since previous studies showed that VPS13 proteins including VPS13D bind and transfer glycerophospholipids and lipid fatty acids moieties in vitro, VPS13D is a potential lipid transfer protein (Kumar et al. 2018; Wang et al. 2021). One possibility is that the lipid transfer activities of VPS13D may be involved in the regulation of the ER-mitochondria interactions. The mechanisms of lipid transfer activities of VPS13D in the regulation of ER-mitochondrial contacts warrant further investigations. In addition, a recent study (Guillén-Samander et al., 2021) show that Rho GTPase Miro1 recruits VPS13D to mitochondria and peroxisomes at ER contacts. Therefore, mitochondrial transport/distribution and cellular Ca2+ dynamics may be involved in the regulation of ER-mitochondrial interactions. All these plausible mechanisms underlying the ER-mitochondrial hyper-tethering resulted from VPS13D suppression are discussed in the discussion section of the revised manuscript.

To make this point clear, we change the title of this manuscript from "VPS13D couples with VCP/p97 to safeguard ER-mitochondria interactions" to "VPS13D interacts with VCP/p97 and negatively regulates ER-mitochondria interactions".

Minor comments

1. On page 4 the authors state "All of these ER-associated MCSs were not substantially altered upon VPS13D suppression". The language here is quite strong. It might be more appropriate to state that the authors did not detect any alterations in ER-associated MCSs.

   Thanks for the suggestion. As suggested, we change the sentence "All of these ER-associated MCSs were not substantially altered upon VPS13D suppression" to "We did not detect any substantial changes in ER-associated MCSs upon VPS13D suppression."

2. At the end of the first paragraph on page 5 (regarding figure S3) the authors could also conclude that the hyper-tethering does not require the presence of VPS13A as they still see hyper-tethering in the VPS13A/D double knockdown condition.

   Thanks for the comment. We add one concluding sentence in the result section of the revised manuscript: "Our results suggested that VPS13A is not required in the ER-mitochondrial hyper-tethering as the hyper-tethering phenotype still exists in the VPS13A/D double knockdown condition."
3. Figure S4 includes panel I which is a replica of Figure 2D. Probably a mistake since S4I is not referenced in the text.

Thanks for catching this. We delete the Figure S4.

4. The authors show convincing double knockdown of VAPB and PTPIP51 in Figure 2D; however, it would also be important to show that VAPB and PTPIP51 are still as efficiently reduced in the VPS13D, VAPB and PTPIP51 triple knockdown cells as in the single KD.

Thanks for the comment. We examined the knockdown efficiency of VAPB and PTPIP51 in the triple knockdown cells. We found that the knockdown efficiency of VAPB and PTPIP51 was almost similar as in the single or double KD cells (Fig. 2E).

5. Page 9: "...VPS13D suppression strongly diminished the localization of p97 puncta at ER-mitochondria MCSs". From the data presented, it is difficult to know whether p97 is specifically diminished from ER-mitochondria MCSs, as opposed to the levels simply being reduced everywhere in the cell (fitting with the data in Figure 3G).

Thanks for the comment. We agree with reviewer that it is difficult to tell whether p97 is specifically reduced at ER-mitochondria MCSs or being reduced all over the cell. Therefore, we change the sentence "VPS13D suppression strongly diminished the localization of p97 puncta at ER-mitochondria MCSs" to "We found a significant decrease in the fluorescence of endogenous p97 protein upon VPS13D suppression (Fig. 3E, middle panel; & 3F), suggesting a correlation between VPS13D and p97 level."

In addition, we moved the immunofluorescence images of endogenous p97 (original Fig. 3E) to Fig. S6A in the revised manuscript, and we removed the quantification result (original Fig. 3F).

6. The error bars in Figure 4C seem non-sensical given that it is a binary phenotype, i.e. cells either do, or do not, exhibit a perturbed mitochondrial morphology. The proper
statistics to use in binary data and contingency tables are for instance the chi-squared or fisher exact tests.

We thank reviewer for the helpful suggestion. As suggested, we re-do the statistics for the Fig. 4C and Fig. S3B by using chi-squared/fisher’s exact test analysis, which are shown below.

![Graph showing statistics](image)

7. The calcium data are interesting but hard to interpret. First, experiments are performed in Opti-MEM, the composition of which is unknown to this reviewer. Most important is the calcium content of this medium. If high (mM range), then Ionomycin most likely causes mitochondrial calcium intake independent of the ER, by permeabilization of the plasma membrane. Either a calcium-depleted medium or a physiological stimulation of IP3 receptor should be used to show increased ER-mitochondria calcium exchange. Second, it isn’t clear at which level the mitochondrial calcium marker saturates. It is quite likely that the absence of large difference in the peak mitochondrial calcium levels are due to saturation of the biosensor.

We thank reviewer for this comment. We agree with reviewer that the Ca^{2+} in medium will complicate our analysis. In addition, thermofisher people can not tell us about the recipe of Opti-MEM (catalog number: 31985062) due to the patent thing. Therefore we decided to remove the calcium data (Fig. 4G and 4F) from the revised manuscript.

8. Fig. 2A shows a rescue of hypertethering by knockdown of VAPB and PTPIP51. It would be interesting to know whether the defects in mitochondrial morphology (Fig 4B-C) are rescued as well. Presumably, this should be easy to quantify from the images the authors already have.

Thanks for the suggestion. As suggested, we quantified the perimeter and distribution of mitochondria. Our quantification shows that both of defects in mitochondrial perimeter
and distribution are significantly rescued upon double knockdown of VAPB and PTPIP51. These results are shown in Fig. 4B and Fig. 4C of the revised manuscript.

9. Having a third of the discussion on the link to lipid droplets seems peculiar when so little of the work presented focuses on lipid-droplets.

We appreciate reviewer for the comment. As suggested, we removed the lipid droplets-related results (Fig. S3G) and removed the discussions regarding lipid droplets in the discussion section of the revised manuscript.
Reviewer #2 (Remarks to the Author):

In their manuscript "VPS13D couples with VCP/p97 to safeguard ER-mitochondria interactions", the authors show that VPS13D controls the extend of contact established between the ER and mitochondria, most probably via altering the levels of the tether VAPB via decreased degradation mediated by p97. Hypertethering induced by depletion of VPS13D caused a substantial enwrapping of mitochondria in ER tubules and affected mitochondrial morphology and distribution, leading to a rather fragmented instead of elongated mitochondrial network. In addition, the authors report on some interesting implications of VPS13D-induced hypertethering for mtDNA replication and mitochondrial calcium handling.

This study is well-done and data and figures are of high quality. The manuscript is well written, and the conclusions drawn by the authors are sound and well justified by presented data and appropriate controls. While a plethora of different tethering components between the ER and mitochondria have been identified in the last years, it is still not clear how these molecular tethers are controlled. This study provides novel insights into the control of ER-mitochondria contact by VPS13D/VAPB/p97, and though the precise molecular events involved are not completely clear, these findings will be interesting for the field.

I only have a few comments and suggestions:

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e.g. via testing whether VPS13D depletion still causes hypertethering when p97 levels are high, e.g. via overexpression? Can some of the phenotypes in respect to ER-mitochondria contact observed upon VPS13D depletion be prevented when p97 levels do not decrease?

We thank reviewer for the questions, and these questions are separately addressed.

1) e.g. via testing whether VPS13D depletion still causes hypertethering when p97 levels are high, e.g. via overexpression?

We examined the ER-mitochondrial interactions in response to p97 overexpression in VPS13D-suppressed U2OS cells. Cells with VPS13D suppression and GFP vector expression substantially increases the ER-mitochondrial connections. Expression of p97-GFP moderately decrease, but not completely rescue, the ER-mitochondrial interaction at both peri-nuclear and periphery regions in VPS13D-suppressed cells (Fig. 3L and N), suggesting that, in addition to the level of p97, unknown pathways may also be responsible for the ER-mitochondrial hyper-tethering phenotype observed in VPS13D suppressed cells.

Consistently, we found that p97 suppression mediated by these two siRNA duplexes significantly enhances the ER-mitochondrial interactions (Fig. 3L and N), but the level is still significantly lower compared to the suppression of VPS13D.

Since previous studies showed that VPS13 proteins including VPS13D bind and transfer glycerophospholipids and lipid fatty acids moieties in vitro, VPS13D is a potential lipid transfer protein (Kumar et al. 2018; Wang et al. 2021). One possibility is that the lipid transfer activities of VPS13D may be involved in the regulation of the ER-mitochondria interactions. The mechanisms of lipid transfer activities of VPS13D in the regulation of ER-mitochondrial contacts warrant further investigations. In addition, a recent study (Guillén-Samander et al., 2021) show that Rho GTPase Miro1 recruits VPS13D to mitochondria and peroxisomes at ER contacts. Therefore, mitochondrial transport/distribution and cellular Ca$^{2+}$ dynamics may be involved in the regulation of ER-mitochondrial interactions. All these plausible mechanisms underlying the ER-mitochondrial hypertethering resulted from VPS13D suppression are mentioned in the discussion section of the revised manuscript.

To make this point clear, we change the title of this manuscript from "VPS13D couples with VCP/p97 to safeguard ER-mitochondria interactions" to "VPS13D interacts with VCP/p97 and negatively regulates ER-mitochondria interactions". 
Can some of the phenotypes in respect to ER-mitochondria contact observed upon VPS13D depletion be prevented when p97 levels do not decrease.

We quantified the mitochondrial perimeter and distribution upon p97 overexpression in VPS13D-suppressed U2OS cells. Our quantification shows that over-expression of p97-GFP significantly restores, but not completely rescue, the defects of mitochondrial fragmentation and distribution in VPS13D-suppressed cells (Fig. 4B and 4C). This result is consistent with the effects of p97-GFP expression on the ER-mitochondrial interactions in VPS13D-suppressed cells.
2. How could VPS13D depletion lead to this drastic drop in p97 levels? Experiments to address this might be beyond the scope of this study, but could the authors provide a section in the discussion to speculate on possible underlying reasons for the strong decrease of p97 when they deplete VPS13D?

This is a great question. We cannot answer the question right now. Our results show that the VAB domain of VPS13D interacted with both the K63-linked and K48-linked ubiquitin chains via in vivo co-immunoprecipitation assays and confocal microscopy (Fig. S5). In addition, we demonstrated that both the UBA domain and the VAB domain interacted with p97 ATPase (Fig. 3B). These results suggested that VPS13D, ubiquitin chains, and p97 might form a ternary protein complex. In considering the contribution of VPS13D to the level of p97, we envision that deletion of one component (VPS13D) may impair the efficient assembly of the ternary protein complex, and unassembled components (p97) may be dislocated or inactive, and may eventually be subject to degradation.

We add our speculation mentioned above in the discussion section of the revised manuscript.

3. VPS13D silencing seems to not only result in increased contact area between ER and mitochondria but to also cause a prominent change in mitochondrial network morphology, leading to a strong fragmentation of mitochondria (Fig. 4). Earlier in the manuscript, the authors show that when they deplete VAPB and PTPIP51, they can prevent hypertethering induced by VPS13D depletion. According to the microscopy pictures shown in Fig. 2F, this also seems to restore an elongated mitochondrial network, though this is hard to judge and the authors did not comment on it. Maybe the authors could consider to additionally quantify mitochondrial perimeter from those pictures, just as they...
have done for VPS13D depletion alone in Fig. 4. This might show that the alterations in mitochondrial morphology and distribution caused by VPS13D depletion are indeed caused by VPS13D-VAPB mediated hypertethering (as hypothesized by the authors in the discussion).

We thank reviewer for this insightful suggestion. As suggested, we quantified the perimeter and distribution of mitochondria. Our quantification shows that both of defects in mitochondrial perimeter and distribution resulted from VPS13D suppression are significantly rescued upon double knockdown of VAPB and PTPIP51. These results are shown in Fig. 4B and Fig. 4C of the revised manuscript.

4. The authors use crude mitochondrial fractions to show that VAPB is more abundant at MAMs/mitochondria when VPS13D is missing. This looks convincing, but it would be good to discriminate between a general increase in VAPB protein levels in total cell lysates and a selective increase of VAPB at the MAMs/mitochondria to see whether VAPB is indeed more efficiently recruited to the contact sites. Does the increase of VABP at MAMs/mitochondria only mirror the increase of VAPB in general (as for instance shown in Fig 3G at the total cellular level) or is it also more prominently recruited to MAMs?

Thanks for the insightful question. We examined the level of Calnexin (a MAMs protein), PTPIP51 (an OMM protein at MAMs), VAPB (a MAMs protein) and COXIV (an IMM protein) in whole cell lysate of scrambled or VPS13D siRNA treated cells. We found that the level of VAPB is increased upon VPS13D suppression in whole cell lysate (WCL),
and the extent of the increase in VAPB level in MAMs/mitochondrial fractions is significantly higher compared to that in WCL (Fig. 2C and 2D).

Minor:

Mostly, statistics seem correct, but for instance in Fig. 2E or S4H, an unpaired student t test was used. However, these are multiple comparisons and thus require the use of a one-way ANOVA to compare between all groups. We thank reviewer for this suggestion. We analyze the Fig. 1B, Fig. 1C, Fig. 2F, Fig. 3K, Fig. 3N, Fig. 4B, and S4H using the one-way ANOVA followed by Tukey’s multiple comparisons test, which are shown below.
The same immunoblots to detect VAPB and PTPIP51 are shown twice (Fig. 2D and Fig. S4I).

Thanks for catching this. We delete the extra blot (Fig. S4I).

Is the coimmunoprecipitation in Fig. 3B lacking a panel to detect UBA-GFP?

Thanks for this comment. We re-do the blots (Fig. 3B), which is shown below. The uncropped blot of VPS13D fragments is also shown.
Fig. 1A shows a very stunning effect of VPS13D silencing on ER-mitochondria contact area in U2OS cells, and the authors refer to Fig. S1A and S1B for respective controls of efficient depletion. However, this now shows an immunoblot for VPS13D depletion in HEK293 cells (S1A) and does not state information about which cell line was used for the RT-qPCR conformation of efficient silencing (Fig. S1B). This needs to be clarified and corresponding controls for the data shown in Fig.1 should be included.

Thanks for the comment. The qPCR results (Fig. S1B) are obtained from U2OS cells, which serve as a control for Fig.1. We add the information to the legend of Fig. S1B in the revised manuscript.
June 1, 2021

RE: Manuscript #E21-03-0097R
TITLE: "VPS13D interacts with VCP/p97 and negatively regulates ER- mitochondrial interactions"

Dear Dr. Ji:

Your revised manuscript has now been seen by the two reviewers; both agree that your changes have improved the study further. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell. Thank you very much for submitting this interesting work to us.

Sincerely,
Martin Ott
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Ji:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
Reviewer #1 (Remarks to the Author):

The revised manuscript and accompanying rebuttal have successfully addressed all concerns with the original data and conclusions. Of particular note, the authors show that the enrichment of VAPB at sites of ER-mitochondria hyper-tethering upon knockdown of VPS13D are specific, and not an artefact arising from the whole ER network being clustered around the mitochondria. Moreover, the authors provide p97 knockdown and overexpression data that support a causal link for p97 in the ER-mitochondria hyper-tethering observed upon loss of VPS13D.

Reviewer #2 (Remarks to the Author):

The authors have revised their manuscript to address the comments raised upon initial evaluation. The authors added a substantial amount of new data, which could strengthen and improve the manuscript. Statistics have been adapted to account for multiple comparisons where appropriate, necessary controls missing in the last version of the manuscript have been included open points have been clarified. All issues raised have been addressed, and I have no further objections but recommend acceptance of this interesting work.

Just a last minor comment: within the new text sections, the authors sometimes switch between present and past tense to describe their work/findings. I am sure this will be corrected prior to publication of the work.