JCB

Mitochondria-ER-PM contacts regulate mitochondrial division and PI(4)P distribution

Jason Casler, Clare Harper, Antoineen White, Heidi Anderson, and Laura Lackner

Corresponding Author(s): Laura Lackner, Northwestern University

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DOI: https://doi.org/10.1083/jcb.202308144

September 25, 2023

Re: JCB manuscript #202308144

Dr. Laura Lackner
Northwestern University
2205 Tech Drive Hogan 2-100
Evanston, IL 60208

Dear Laura,

Thank you for submitting your manuscript entitled "Mitochondria-ER-PM contacts regulate PI(4)P distribution and mitochondrial division". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that the reviewers appreciate that your study examines the different functions of mitochondrial-ER, mitochondrial-PM, and ER-PM tethers. In addition, the concomitant effect on PI(4)P distribution and mitochondrial division is an intriguing observation. However, we agree with the opinion of reviewer 2, which was also an initial editorial concern, that to be suitable for the broad readership of JCB the study needs to provide more mechanistic insight into the PI(4)P phenotype. The first two points of reviewer 2 address the key questions that should be considered for further consideration at JCB. Therefore, although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal and our journal office will transfer the reviews at your request.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that provide significant mechanistic insight into how Num1 affects PI(4)P distribution in the plasma membrane. This manuscript would be subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to submit a revision plan which may be discussed with the reviewers to help ensure that we are all in agreement about the expectations for a resubmission to JCB. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Thomas Langer
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The manuscript by Casler et al uncovers a new function for the three-way (mitochondria-ER-PM) contact site termed the MECA in yeast. They continue to demonstrate that this function, in regulating PI4P distribution on the PM, also has secondary effects on mitochondrial dynamics. The authors beautifully dissect the mechanism of Num1, the first discovered tether for this contact, in this activity and find that it binds to the VAP like protein Scs2 on the ER through a FFAT motif. This allows them to clearly distinguish between Num1s various functional roles. This is a carefully performed and beautiful dissection of an important regulatory node at a contact site. Using a plethora of synthetic tools, careful imaging and quantitation and accurate mutagenesis they have managed to accurately assign functions to the various complex activities of the Num1 protein. As such it is highly worthy of publication in JCB. I do have several suggestions that would make the manuscript more accurate and more readable to the general cell biology community:

Large requests

1. Figure I panel C/D - I do not see any difference in cortical ER in the Δlnp1 vs the LNP1 WT cells. This may be the specific choice of panels but it could also be that this Δlnp1 background is not really that impactful on cortical ER structure. If the latter is the case I think that for the sake of simplicity the authors should remove all of the Δlnp1 data. I fail to see in any of the figures where this is used that this is really helpful - in my eyes the WT cells already show a difference that is convincing (similarly in figure 2D/E and 4B/C/D).
2. Panel 1E please mark on the panel (and not just in the figure legends) what is the statistical comparison made to.
3. Figure 3D - there is a difference in average - is this statistically significant?
4. For Figure 3E/F - how many cells were tracked? Was the phenotype seen consistent in 100% of cells tracked? Please write this in the figure legend.
5. Figure 3I the authors write "our results demonstrate that the Num1-Scs2 interaction is not required .... to alleviate tunicamycin-induced ER stress" however even Δnum1 has no role in alleviating tunc. induced stress hence I think this data is not relevant for the manuscript and is, at present, written in a confusing way. I suggest to remove this data altogether.
6. I do not see how figure 4B is different (conceptually) than figure 1D. There is no need to show this experiment again and the authors can simply refer back to the figure already shown. If the issue is the "top view" than it can be in supplementary materials and does not need to be again in the main figures.
7. In general figure 4 is presented in a confusing way with not all experiments having their controls in the same figures. In my eyes, to check if the Num1-Scs2 interaction is of sufficient strength to alter ER-eisosome associations (as the authors state) they need a clear set of experiments shown together - the Lsp1-mKATE /Tcb3-GFP overlap in PAN cells with WT NUM1, Δnum1, Num1ΔFFAT so that they are comparable.
8. In 4C why did the authors use Δph and not wt NUM1?
9. This is in no way necessary but if the authors wish to better demonstrate the requirement for transcriptional rewiring to support PI4P distribution upon reintroduction of the contact (Figure 7) they might consider repeating the RID experiment with CHX addition and showing that no normalization of PI4P distribution is obtained.

Small textual requests
1. In row 46 the authors state "Scs2 and Scs22, homologs of the mammalian VAP proteins, are integral membrane proteins that function as two of six known ER-PM tethers in yeast" however I think the count has grown since discovery of the LAM proteins that are also tethers at the ER-PM contact. This should be altered accordingly and the manuscripts of the relevant LAMs cited.

Reviewer #2 (Comments to the Authors (Required)):

Casler et al in this Ms report on the role of the yeast protein Num1 in multiorganellar tethering of the ER and mitochondria to the plasma membrane via tripartite membrane contacts (referred to as MECA) and its effects on plasma membrane PI(4)P distribution and mitochondrial fission. While the mechanisms that tie the core MECA scaffold protein Num1 to mitochondria and the PM have been established, less is known regarding the alleged association of Num1 with the cortical ER. The authors use confocal imaging in yeast different genetically engineered yeast strains to demonstrate that Num1 associates with the ER by association with the VAP homolog Scs2 via an FFAT motif in its C-terminal domain. Loss of the ER tethering function of Num1 did not affect its role as a mitochondrial tether for the PM or as an anchor for dynein during nuclear inheritance. Instead, loss of Num1-mediated ER tethering affected the distribution of PI(4)P on the PM in dividing cells, possibly by altering the distribution of the PM PI 4-kinase complex comprising Stt4, Ypp1, and Efr3. Elegant experiments utilizing ligand-induced reconstitution of Num1 function showed that mitochondria-ER-PM contacts indeed are required and sufficient to restore the normal distribution of PI(4)P in Num1 KO cells. Finally, it is shown that loss of Num1-Scs2 contacts causes defects in mitochondrial division that surprisingly do not result in altered mitochondrial morphology. Based on the results a model for the function of Num1 in regulating the distribution of PI(4)P on the PM via tripartite membrane contacts is presented.

The Ms is a follow-up from previous works suggesting a role for Num1 in MECA formation or stability. Aside from the mapping of the interaction site within Num1 for Scs2 the main novelty of this study lies with the observed phenotype on PI(4)P distribution on the PM, e.g. the partitioning between the bud and the mother cell. These experiments are well thought-out and generally compelling, although the mechanism by which Num1 and Scs2 regulate PI(4)P distribution on a time scale of hours rather than minutes remains to be determined. If such mechanism was provided, the study would present a major leap in our understanding of the roles of ER/PM contacts and the possible role of mitochondria in controlling PI(4)P and, possibly, other PM lipids.

I have several major points that need to be addressed prior to considering the study for publication in the JCB.

1. The authors provide convincing evidence that deletion of the FFAT motif in Num1 or loss of Scs2 impair the concentration of PI(4)P on the PM of the bud in dividing yeast cells. However, I miss any data regarding the overall levels of PI(4)P in deltaFFAT expressing cells or cells lacking Num1 altogether. From the author's model it seems as if they favor a role for MECA in PI(4)P hydrolysis via lipid transfer to Sac1 but no evidence is provided. This is a key point that should be addressed experimentally.

2. A further loose end of the data pertains to the alleged weak association of Num1 with the PI4K complex. Apart from the proteomic data that remain insignificant, it remains unclear how Num1 affects the Ypp1-Efr3-Stt4 complex. Does loss of Num1 or

its FFAT motif alter Ypp1-Efr3-Stt4 complex assembly? Does Num1 bind to the Ypp1-Efr3-Stt4 complex directly or indirectly (e.g. at the level of Co-Ips, proximity biotinylation, BiFC etc.)? Is Stt4 activity altered in Num1 KO or deltaFFAT expressing cells?

3. Although, loss of the Num1-Scs2 interaction did not perturb mitochondrial morphology (see Fig3), the authors report a defect in mitochondrial division in Num1deltaFFAT cells. How is mitochondrial fusion affected in Num1deltaFFAT cells? Is the expression or localization of mitochondrial fusion and fission factors such as Dnm1, Fzo1 etc affected by loss of Num1 or in Num1deltaFFAT?

Other points:
4. The co-ip data shown in Fig.2B,C do not rise to the standard expected for a JCB paper. Better data are needed to support complex formation between Num1 and Scs2 in yeast.

5. Fig. 6A misses representative images.

6. Fig. S4F: I am not convinced by the images presented and would like to see better evidence that the artificial PM-mitochondrial tethers indeed restore cortical mitochondrial networks.

Northwestern | Department of Molecular Biosciences
WEINBERG COLLEGE OF ARTS & SCIENCES

Laura Lackner, Ph.D.
Associate Professor

2205 Tech Drive, Hogan 2-100
Evanston, IL 60208-3500

April 25, 2024

Dear Drs. Langer and Marat,

Thank you for overseeing the review of our manuscript (#202308144). The reviewers' and editors' comments were constructive and helped to strengthen the manuscript considerably. As you will see, we have significantly revised and extended the manuscript to address the concerns raised.

The primary concern with the initial submission was a lack of mechanistic insight into how MECA regulates PI(4)P metabolism. To address this, we introduce several lines of evidence that Num1 influences Sac1-mediated PI(4)P turnover. We demonstrate that loss of Num1 or Scs2 does not affect the localization of PIK patch components, suggesting that the function of the PI4Kinase is not disturbed (revised Fig. S5). In addition, we developed a system to measure the turnover of PM-localized PI(4)P. Upon conditional inactivation of PIK patches by degrading Efr3, the PM PI(4)P pool is rapidly lost, presumably through Sac1-dependent PI(4)P turnover. Loss of PM PI(4)P was markedly slower when this experiment was performed in a *num1*Δ strain (revised Fig. 8). To further address mechanism, we provide evidence that both reducing or enhancing the Num1-Scs2 interaction alters PI(4)P distribution (revised Fig. 6 and 7). These data suggest that the optimal arrangement of Num1 clusters may locally regulate Scs2-FFAT motif interactions and the organization of PIK patch components in a manner that favors efficient PI(4)P turnover. Finally, we also performed experiments that suggest an explanation for why Num1 apparently influenced PI(4)P distribution over longer timescales than anticipated. By using 4D confocal microscopy to track the distribution of PI(4)P throughout the cell cycle, we determined that PI(4)P polarity is progressively lost as cells approach cytokinesis (revised Fig. 9). We then re-performed our RID-Num1 rescue experiments and tracked when PI(4)P polarity was established. The data suggest that the rescue is likely fairly rapid, with most newly budded cells establishing a PI(4)P polarity that resembles wild type. We propose a model in which the progressive formation of Num1-mediated mitochondria-ER-PM contact sites in the growing bud coincides with an increase in Sac1-mediated PI(4)P turnover and reduction in PI(4)P polarity. In addition, we were able to address the vast majority of the other major and minor reviewer comments and we have attached a point-by-point response.

Thank you,

Responses to Reviewer's Comments

We have added new experiments to address the reviewers' comments and have modified figures based on reviewer suggestions. These additions and changes resulted in significant changes to the results, discussion, and figures. We have done our best to highlight all changes to the revised manuscript in blue. We have also described the changes and additions in our responses below.

Reviewer #1

General Comments: *The manuscript by Casler et al uncovers a new function for the three-way (mitochondria-ER-PM) contact site termed the MECA in yeast. They continue to demonstrate that this function, in regulating PI4P distribution on the PM, also has secondary effects on mitochondrial dynamics. The authors beautifully dissect the mechanism of Num1, the first discovered tether for this contact, in this activity and find that it binds to the VAP like protein Scs2 on the ER through a FFAT motif. This allows them to clearly distinguish between Num1s various functional roles. This is a carefully performed and beautiful dissection of an important regulatory node at a contact site. Using a plethora of synthetic tools, careful imaging and quantitation and accurate mutagenesis they have managed to accurately assign functions to the various complex activities of the Num1 protein. As such it is highly worthy of publication in JCB. I do have several suggestions that would make the manuscript more accurate and more readable to the general cell biology community:*

We thank the reviewer for their very positive and insightful comments and have addressed the majority of the requests as outlined below. In doing so, we feel we have strengthened the manuscript.

Large requests

1. *Figure I panel C/D - I do not see any difference in cortical ER in the ∆lnp1 vs the LNP1 WT cells. This may be the specific choice of panels but it could also be that this ∆lnp1 background is not really that impactful on cortical ER structure. If the latter is the case I think that for the sake of simplicity the authors should remove all of the ∆lnp1 data. I fail to see in any of the figures where this is used that this is really helpful - in my eyes the WT cells already show a difference that is convincing (similarly in figure 2D/E and 4B/C/D).*

   We appreciate the reviewer's comments to try to simplify the microscopy used to demonstrate ER-localization. In line with several other of the reviewer's comments, we have significantly streamlined the beginning of the manuscript and eliminated much of the *lnp1*∆ microscopy data. We have kept the *lnp1*∆ data in the truncation analysis (revised Fig. 1B), the PAN experiments (revised Fig. 2), and the 3 color movies of Num1, mitochondria, and ER (revised Fig. 3, E and F) as we believe the altered ER-morphology in *lnp1*∆ cells more clearly shows the indicated ER-localization phenotypes. Use of mutants to disrupt ER-morphology to emphasize ER-localization are fairly common, and similar experiments have been performed with reticulon (*rtn1*∆ *rtn2*∆ *yop1*∆) mutants (Stefan et al., 2011).

2. *Panel 1E please mark on the panel (and not just in the figure legends) what is the statistical comparison made to.*

   We have tried to include indicators of statistical comparisons in all graphs where appropriate.

3. *Figure 3D - there is a difference in average - is this statistically significant?*

The difference in mean is not statistically significant and is now indicated in the figure.

4. *For Figure 3E/F - how many cells were tracked? Was the phenotype seen consistent in 100% of cells tracked? Please write this in the figure legend.*

   We have included the following quantification of the microscopy data in the text:

   "In 98% of cells expressing full length Num1, all Num1 foci were associated with both the ER and mitochondria (n = 100 cells from 3 movies). In contrast, in cells expressing Num1ΔFFAT, the percent of cells in which all Num1 foci were associated with both the ER and mitochondria decreased to 21%; the other 79% of cells contained at least one Num1ΔFFAT foci that was clearly spatially separated from the ER (n = 100 cells from 3 movies)."

5. *Figure 3I the authors write "our results demonstrate that the Num1-Scs2 interaction is not required .... to alleviate tunicamycin-induced ER stress" however even ∆num1 has no role in alleviating tunc. induced stress hence I think this data is not relevant for the manuscript and is, at present, written in a confusing way. I suggest to remove this data altogether.*

   We agree with the reviewer and have removed the tunicamycin growth assays.

6. *I do not see how figure 4B is different (conceptually) than figure 1D. There is no need to show this experiment again and the authors can simply refer back to the figure already shown. If the issue is the "top view" than it can be in supplementary materials and does not need to be again in the main figures.*

   We agree that the indicated experiments were redundant. In the revised manuscript, a comparison between the localization of ER and eisosomes is only present in Fig. 2B.

7. *In general figure 4 is presented in a confusing way with not all experiments having their controls in the same figures. In my eyes, to check if the Num1-Scs2 interaction is of sufficient strength to alter ER-eisosome associations (as the authors state) they need a clear set of experiments shown together - the Lsp1-mKATE /Tcb3-GFP overlap in PAN cells with WT NUM1, ∆num1, Num1∆FFAT so that they are comparable*

   We agree with the reviewer that the original organization of these experiments was not optimal. In the revised manuscript, all relevant ER-eisosome localization experiments are contained in Fig. 2 with additional representative microscopy images present in Fig. S1. Fig. 2 C and D now demonstrate that expression of PAN alters ER-eisosome associations, and Fig. 2 E, F, and H demonstrate that this is dependent on the Num1-Scs2 interaction.

8. *In 4C why did the authors use ∆ph and not wt NUM1?*

   The Num1 PH domain is required to target Num1 to the PM. In order to restrict the formation of cortical Num1 clusters to eisosomes, we remove the PH domain to force PM-localization through the interaction between Num1ΔPH-GFP and Pil1- αGFP. To help clarify this point, we have changed the figure labels to indicate that PAN or PANΔFFAT clusters are being visualized.

9. *This is in no way necessary but if the authors wish to better demonstrate the requirement for transcriptional rewiring to support PI4P distribution upon reintroduction of the contact (Figure 7)they might consider repeating the RID experiment with CHX addition and showing that no normalization of PI4P distribution is obtained.*

We appreciate the suggestion and would like to note that we have added new experiments to the manuscript that provide a plausible explanation for why the rescue of PI(4)P distribution takes place significantly after the inducible MECA contact sites are formed. In the process of doing experiments to better understand the delay in rescue, we made the exciting observation that PI(4)P distribution on the PM is regulated with the cell cycle. Specifically, we found that a polarized distribution of PI(4)P is observed in small budded cells, is lost by the time of cytokinesis, and is reestablished upon growth of a new bud (see revised Fig. 9). Given that rapamycin is added to an asynchronous cell population, it will take 1-3 hours for all cells in the population to undergo a full cell cycle, which likely explains the gradual rescue observed in our initial experiments (Fig. 9, C and D). Thus, the rescue likely occurs rapidly, but can be difficult to measure in bulk populations as PI(4)P polarity is regulated throughout the cell cycle.

Small textual requests

1. *In row 46 the authors state "Scs2 and Scs22, homologs of the mammalian VAP proteins, are integral membrane proteins that function as two of six known ER-PM tethers in yeast" however I think the count has grown since discovery of the LAM proteins that are also tethers at the ER-PM contact. This should be altered accordingly and the manuscripts of the relevant LAMs cited.*

   We thank the reviewer for pointing out these important studies. We have included a reference to Quon et al., which identified Ice2 as an addition ER-PM tether.

Reviewer #2

General comments: *Casler et al in this Ms report on the role of the yeast protein Num1 in multiorganellar tethering of the ER and mitochondria to the plasma membrane via tripartite membrane contacts (referred to as MECA) and its effects on plasma membrane PI(4)P distribution and mitochondrial fission. While the mechanisms that tie the core MECA scaffold protein Num1 to mitochondria and the PM have been established, less is known regarding the alleged association of Num1 with the cortical ER. The authors use confocal imaging in yeast different genetically engineered yeast strains to demonstrate that Num1 associates with the ER by association with the VAP homolog Scs2 via an FFAT motif in its C-terminal domain. Loss of the ER tethering function of Num1 did not affect its role as a mitochondrial tether for the PM or as an anchor for dynein during nuclear inheritance. Instead, loss of Num1-mediated ER tethering affected the distribution of PI(4)P on the PM in dividing cells, possibly by altering the distribution of the PM PI 4-kinase complex comprising Stt4, Ypp1, and Efr3.Elegant experiments utilizing ligand-induced reconstitution of Num1 function showed that mitochondria-ER-PM contacts indeed are required and sufficient to restore the normal distribution of PI(4)P in Num1 KO cells. Finally, it is shown that loss of Num1-Scs2 contacts causes defects in mitochondrial division that surprisingly do not result in altered mitochondrial morphology. Based on the results a model for the function of Num1 in regulating the distribution of PI(4)P on the PM via tripartite membrane contacts is presented.*

The Ms is a follow-up from previous works suggesting a role for Num1 in MECA formation or stability. Aside from the mapping of the interaction site within Num1 for Scs2 the main novelty of this study lies with the observed phenotype on PI(4)P distribution on the PM, e.g. the partitioning between the bud and the mother cell. These experiments are well thought-out and generally compelling, although the mechanism by which Num1 and Scs2 regulate PI(4)P distribution on a time scale of hours rather than minutes remains to be determined. If such mechanism was provided, the study would present a major leap in our understanding of the roles of ER/PM contacts and the possible role of mitochondria in controlling PI(4)P and, possibly, other PM lipids.

I have several major points that need to be addressed prior to considering the study for publication in the JCB.

We thank the reviewer for the overall positive comments on the manuscript and data. As described in our intro paragraph to the editors, in our response to Reviewer #1 Large Request #9, and in our responses below, we have provided additional mechanistic insight into how MECA may regulate PI(4)P metabolism. In the process of carrying out experiments to address mechanism, we made the exciting observation that PI(4)P distribution on the PM is regulated with the cell cycle. The change in polarization of PI(4)P over the cell cycle provides a plausible explanation for why the rescue of PI(4)P distribution takes place significantly after the inducible MECA contact sites are formed (see response to Reviewer #1 Large Request #9).

Major points

1. *The authors provide convincing evidence that deletion of the FFAT motif in Num1 or loss of Scs2 impair the concentration of PI(4)P on the PM of the bud in dividing yeast cells. However, I miss any data regarding the overall levels of PI(4)P in deltaFFAT expressing cells or cells lacking Num1altogether. From the author's model it seems as if they favor a role for MECA in PI(4)P hydrolysis via lipid transfer to Sac1 but no evidence is provided. This is a key point that should be addressed experimentally.*

   We agree with the reviewer and editors that the original manuscript lacked evidence that MECA influences Sac1-mediated PI(4)P hydrolysis. To address this, we developed a system to measure the turnover of PM-localized PI(4)P. Upon conditional inactivation of PIK patches by degrading Efr3, the PM PI(4)P pool is rapidly lost, presumably through Sac1-dependent PI(4)P turnover (Fig. 8). Loss of PM PI(4)P was markedly slower when performing this experiment in a *num1*Δ strain. This result is consistent with a mechanism in which Num1 influences the efficiency of Sac1-mediated PI(4)P hydrolysis.

   Unfortunately, the direct quantification of PI(4)P levels is not trivial. Our group is currently unequipped to perform such experiments and we are unaware of a commercial service that could provide the requested measurements. We would like to note, however, that other groups have measured overall PI(4)P levels in Scs2 mutants, and found them to be higher than wild type (Stefan et al., 2011). Our evidence suggests Num1 influences PI(4)P metabolism through the same mechanism as Scs2. Thus, it seems likely that loss of Num1 would have a similar, but weaker effect compared to the loss of Scs2. The primary phenotype that we characterized is a shift in the relative enrichment of PM PI(4)P between the bud and mother. As such, we believe the comparative measurements between the two membranes at a single cell level are more informative than an absolute quantification of the amount of PI(4)P. Therefore, while certainly of interest and a direction for future research, we believe the requested measurements are beyond the scope of this study.

2. *A further loose end of the data pertains to the alleged weak association of Num1 with the PI4Kcomplex. Apart from the proteomic data that remain insignificant, it remains unclear how Num1 affects the Ypp1-Efr3-Stt4 complex. Does loss of Num1 or its FFAT motif alter Ypp1-Efr3-Stt4complex assembly? Does Num1 bind to the Ypp1-Efr3-Stt4 complex directly or indirectly (e.g. at the level of Co-Ips, proximity biotinylation, BiFC etc.)? Is Stt4 activity altered in Num1 KO or deltaFFAT expressing cells?*

   We would like to thank the reviewer for suggesting these important experiments to further clarify the Num1-PIK patch interaction. We have included the following experiments in the revised manuscript:

a. Num1 is detected in co-IP experiments with Efr3 (revised Fig. 5 F). The interaction may be indirect, as loss of the Num1 FFAT motif reduces colocalization of Num1 and PIK patches (revised Fig. 5, D and E).
b. Num1 and Scs2 do not obviously influence the localization of Efr3, Ypp1, Stt4, or Sac1 (revised Fig. S5, C-E).
c. Conditional inactivation of PIK patches by degrading Efr3 causes a loss of PI(4)P from the PM (revised Fig. 8). PI(4)P is clearly present on the PM when either Num1 or Scs2 is absent (revised Fig. 5, H and J). Therefore, it is unlikely that Stt4 activity is strongly negatively impacted in these conditions. It is possible Stt4 activity could be increased in these mutants, although that seems unlikely.

3. *Although, loss of the Num1-Scs2 interaction did not perturb mitochondrial morphology (see Fig3), the authors report a defect in mitochondrial division in Num1deltaFFAT cells. How is mitochondrial fusion affected in Num1deltaFFAT cells? Is the expression or localization of mitochondrial fusion and fission factors such as Dnm1, Fzo1 etc affected by loss of Num1 or in Num1deltaFFAT?*

   We appreciate the suggestion of this important control. We quantified mitochondrial fusion rates from the same cells where mitochondrial division was previously analyzed. Mitochondrial fusion rates are perturbed to a similar degree as mitochondrial fission rates when the Num1-Scs2 interaction is lost (revised Fig. 4, A-C). Additionally, neither Dnm1 nor Fzo1 have any obvious localization defects (revised Fig. S3). Due to the increase in cells showing netted mitochondrial networks upon expression of the hypomorphic Dnm1-GFP allele, we believe the primary effect of losing the Num1-Scs2 interaction is on mitochondrial division (revised Fig. 4, D and E).

Other points

1. *The co-ip data shown in Fig.2B,C do not rise to the standard expected for a JCB paper. Better data are needed to support complex formation between Num1 and Scs2 in yeast.*

   We have re-performed the co-IP analysis of Num1 and Scs2 and included an additional point mutant, Num1(F2135A), that also disrupts the Num1-Scs2 interaction. All data included in the quantification are from new co-IP experiments of similar quality to the representative blot shown.

2. *Fig. 6A misses representative images.*

   We have included representative images in the revised manuscript.

3. *Fig. S4F: I am not convinced by the images presented and would like to see better evidence that the artificial PM-mitochondrial tethers indeed restore cortical mitochondrial networks.*

   We are happy to provide additional images if the reviewer finds them to be necessary; however, these artificial tethering systems have been extensively characterized in a recent publication from our group (White et al., *Journal of Cell Science* 2022). Both artificial tethers restore mitochondria-PM tethering to levels similar to or higher than wild type Num1-mediated mitochondrial tethering (White et al., Fig. 3, B and C). In addition, Num1ΔFFAT restores cortical tethering of mitochondria but does not rescue PI(4)P distribution, which is consistent with our reasoning that mitochondria-PM tethering alone is not sufficient to maintain the normal distribution of PI(4)P on the PM.

April 29, 2024

RE: JCB Manuscript #202308144R-A

Dr. Laura L Lackner
Northwestern University
2205 Tech Drive Hogan 2-100
Evanston, IL 60208

Dear Laura,

Thank you for submitting your revised manuscript entitled "Mitochondria-ER-PM contacts regulate mitochondrial division 1 and PI(4)P distribution". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. To ensure clarity to color blind readers the use of red/green in images and graphs is discouraged.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed.

7) All antibodies, cell lines, animals, and tools used in the manuscript should be described in full, including accession numbers for materials available in a public repository such as the Resource Identification Portal. Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model

g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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Reviewer #2 (Comments to the Authors (Required)):

All my previous comments and questions have been addressed appropriately. I suggest acceptance of the Ms for publication in the JCB.