Loss of Num1-mediated cortical dynein anchoring negatively impacts respiratory growth

Antoineen J. White, Clare S. Harper, Erica M. Rosario, Jonathan V. Dietz, Hannah G. Addis, Jennifer L. Fox, Oleh Khalimonchuk and Laura L. Lackner
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MS TITLE: Unraveling the role of the multifunctional mitochondria and dynein anchor Num1 in mitochondrial function

AUTHORS: Antoineen J White, Clare S Harper, Erica M Rosario, Jonathan V Dietz, Hannah G Addis, Jennifer L Fox, Oleh Khalimonchuk, and Laura L Lackner
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Membrane contact sites serve as regions of material exchange between compartments and membranes, and play important roles in the placement of organelles within the cell. The MECA contact site formed by its main tether Num1 is important for mitochondrial morphology and for retention of mitochondria in the mother cell during cell division. In addition, Num1 plays a role in anchoring dynein to the cell cortex, a process required for proper nuclear inheritance.
The manuscript by White et al reports that lack of cortical anchoring of dynein by Num1 produces defects in respiratory growth. The defective respiratory growth is not causes by lack of functional anchoring of Dynein, but rather by a toxic effect of the misplaced protein, as it can be suppressed by deletion of Dyn1.
They arrive at this conclusion by analyzing the effect on a number of separation-of function mutants of Num1 and artificial tethers of the mitochondria-cortex contact site. The phenotype that they report is interesting as it adds a new layer to the function of Num1, suggesting that the anchoring of Dynein at the cortex is not only important for its role in nuclear migration but also for preventing a specific toxicity of the protein for respiratory growth.
I think the study would represent a significant advance in the field and be thus suitable for publication, only if the authors show some insight into how the mislocalization of Dynein affects mitochondrial function and respiratory growth.

Comments for the author

1) The phenotype reported is interesting, but the study lacks any mechanistic insight into why lack of Dynein anchoring results in lack of respiratory growth. The authors discuss a few possibilities, including an effect on stability of microtubules or a direct interaction of the protein with mitochondria, but no experiments are made to explore these possibilities. In my opinion, the article would only represent a significant advance in the field that would warrant publication if they provide some insight into why does Dynein mislocalization cause mitochondrial dysfunction. Some ideas on how this could be approached:
- What about the other components of dynein? Dyn3 does not localize to the cortex but is required for Dyn1 localization at the cortex (Lee et al, JCB 2005). Would deletion of Dyn3 result in the same phenotype regarding mitochondrial function as deletion of Num1?
- Would overexpression of Dyn1 have a similar effect? (by surpassing the available Num1 binding sites)
- Is Dynein binding to microtubules required for the toxicity? Would deleting proteins required for delivering Dynein to the plus end rescue the toxicity? (bik1-CTΔ40, see Sheeman et al, Current Biology, 2003)
- Does Dyn1 interact with mitochondria under these conditions? Options to do this would be fluorescence microscopy, or if this is hard to achieve as the authors state in the discussion, alternatives would be biochemical assays:
o Co-purification of Dynein with mitochondria under these conditions.
o Pull down experiments to detect the binding partner on mitochondria o BioID to detect the molecular microenvironment.
- Could they perform a screen to find mutants of Dyn1 which are not toxic under these conditions?

2) They show many assays in which they quantify colony size and many assays where they quantify the fraction of the cell cortex containing mitochondria. In these assays they show data for individual colonies/cells, from at least 3 independent experiments according to Figure legends. However, the data is shown as just one set. They should separate the data from each experiment in some way
(either side by side or color coding), and show the average of each experiment.
In this way, the figures would not only depict the biological variability of the system but also the reproducibility.
In addition, statistical comparisons should be shown, to see if the observed differences are significant. Importantly, the statistical comparisons should be done using the avarages of the experiments, and not all the single cell/colony data. More information on how to depict and perform statistical comparisons on such data is discussed in Lord, Velle, Mullins and Fritz-Laylin,

JCB, 2020 3) I would suggest a title that states the finding of the paper in a more straightforward manner.

Reviewer 2

Advance summary and potential significance to field

This study examines the role of the multifunctional mitochondrial tether Num1 in mitochondrial spatial organization and cell growth. Num1 is characterized as a mitochondrial-PM tether, but also interacts with the cytoskeleton to control organelle organization and inheritance. This study investigates the relative importance of each of these Num1 interactions and how they relate to yeast growth and respiration. Through elegant structure-function analysis, Num1 targeting to the PM and its associated mitochondrial polarization to the cell surface is found to influence yeast growth and respiration. Loss of Num1 leads to reduced cell growth and respiration, but this can be rescued through artificial Num1 fragments that target to the eisosomes on the PM.
However, all artificial tethers required the Num1 CC domain, suggesting another role for the CC domain other than simply mitochondrial tethering was required for Num1 function. Surprisingly experiments suggest that the CC domain's role in positioning the dynein complex at the cell surface, which aids in yeast nuclear inheritance during budding, is the key role for Num1. In support of this, artificially delivering Dyn1 to the PM via an artificial anti-GFP nanobody strategy can rescue growth defects in Num1KO. The study indicates that the growth phenotype observed in Num1-deleted yeast is primarily due to mislocalized dynein rather than defective mitochondrial-PM contact.

Comments for the author

The study appears rigourous and the experiments are well controlled and interpreted. The model, that loss of Num1 mitochondrial tethering is not the causative effect of the Num1 growth defect, is surprising. The alternative model, that Dyn1 mislocalization is the true culprit, is well investigated, although somewhat underexplored at the end of the study. The only significant concern would be that the study does not offer a more direct explanation for why mis-localized Dyn1 causes defects in growth. Following up on this with a few characterizing experiments would enhance the already thorough study.

Major concerns:
1.The study indicates that the mislocalization of Dyn1, rather than Num1 mito-PM tethering or nuclear inheritance, is causative of the growth and respiration defects observed in Num1 KO. What issue does having mislocalized Dyn1 have on yeast and mitochondrial health? Since the Dyn1 is likely remaining on microtubules does this effect microtubule dynamics or other trafficking events?
2. Similar to the point above, how does Num1 loss impact Dyn1 abundance? Can manipulating Dyn1 levels through AID or over-expression also bypass Num1 loss?
3. There is a general lack of statistical analysis for some of the panels. For example Fig 3D, Fig 4A. Please provide statistical tests and information.

Reviewer 3

Advance summary and potential significance to field

The manuscript by White et al dissected the role of Num1 in mitochondrial homeostasis. This is complex since Num1 is has two main functions: the first in tethering the Plasma membrane-mitochondria-ER three-way contact site and the second in nuclear inheritance to the bud. The authors found that the CC domain of Num1 is the one required for mitochondrial function. Using synthetic tethering and growth assays they nicely showed that the effect on mitochondria was not due to either reduction in contact site or defects in nuclear inheritance. Rather, and quite surprisingly, it is the misregulation of cortical dynein anchoring that is important for proper mitochondrial function. This study is a beautiful example of how mechanistic dissection of a biological process should be performed and is well-executed and quantified. Most importantly, it broadens our knowledge regarding the multifunctional Num1 protein, dynein regulation and

mitochondrial function and is therefore highly worthy of publication in JCS following consideration of the comments stated below:

Comments for the author

Textual:
1. Line 155: ".. crucial functions of mitochondria is the production of energy …".
Please rephrase the sentence since mitochondria do not produce energy (a physical impossibility), but rather produce ATP molecules.
2. We suggest to move Figure 1F to supplementary figures since mdm36 and dyn1 mutations are not the main focus of the paper. There is no view of Num1 mutants in these conditions and is merely a control for the panel coming before it.
3. We suggest to make a new "Results" section with its own header for the text describing the results in Figure 3 (that right now is continuous with the text describing Figure 2). This will help highlight this important section.
4. We suggest to add a schematic illustration of the final mechanism that is described in the discussion section (lines 415-416) as a closing model Figure.
Experimental:
1. Figure 1C- The figure shows higher expression levels of Num1 in respiratory condition compared to fermentation condition. While the authors show "whole protein" levels in the Supplementary as proof of equal loading – this is not sufficient for quantitation. We suggest addition of a loading control such a housekeeping protein and quantitation of this blot.
2. Figure 1D - The fermentation condition are confusing since they were used to normalize everything to so, of course, the fold change is 1. We suggest to remove this bar and leave only the respiration.
3. Loss of Mdm36 is suggested not to have any effect on growth (Figure 1).
However, Mdm36 is a hypomorph of Num1 so it is clear that it will have less effect. The temperature that was used in this experiment was 35OC, however, in this condition Num1 shows little effect. Therefore, to really state this the growth rate of Δmdm36 strain should be measured in 37OC – conditions in which loss of Num1 had a dramatic effect.
4. Figure 2B- Two important controls are missing- Full length Num1 tagged with GFP on its C terminus to check that the GFP tagging is not the cause of the growth defect in respiratory condition since all the different strains were tagged with GFP. Another important control is tagging only the PH domain of Num1 with GFP. It is possible to tag one of the mitochondrial membrane proteins such as Tom20/Tom70 with αGFP and observe if this synthetic tethering can rescue the phenotype.
5. All graphs presented in this paper should show the P values or other statistical significance measurement between the different strains compared.

First revision

Author response to reviewers' comments

Response to Reviewers' Comments:
We thank the reviewers for their very positive and insightful comments. We have revised the manuscript based on the comments and suggestions and feel the manuscript has been strengthened by the revisions. We have been able to successfully address the vast majority of the reviewer comments and have provided a detailed response to each of the reviewers' comments below. We have added experiments that further investigate the mechanism by which unanchored dynein negatively impacts mitochondrial function. Specifically, by analyzing a variety of dynein and dynein pathway mutants, we provide evidence indicating that the full dynein complex and its ability to interact directly with microtubules is required for the defect in respiratory growth observed in cells lacking Num1. To gain a complete understanding of the mechanism will take time to complete and is beyond the scope of the current study.

We strongly feel that our revised manuscript provides a substantial and unexpected advance in our understanding of mitochondria-dependent dynein anchoring. Previous work has demonstrated that

mitochondrial tethering impacts dynein-mediated nuclear migration. Here, we extend the functional connection between mitochondria and dynein and find that the inability to anchor dynein at MECA sites negatively impacts mitochondrial function. Thus, we provide further support for the role of the multifunctional MCS, MECA, as a hub that impacts and integrates the spatial organization and function of organelles within the cell.

In the revised manuscript document, all modifications to the text have been highlighted using blue text.

Reviewer 1

Advance Summary and Potential Significance to Field*: Membrane contact sites serve as regions of material exchange between compartments and membranes, and play important roles in the placement of organelles within the cell. The MECA contact site formed by its main tether Num1 is important for mitochondrial morphology and for retention of mitochondria in the mother cell during cell division. In addition, Num1 plays a role in anchoring dynein to the cell cortex, a process required for proper nuclear inheritance. The manuscript by White et al reports that lack of cortical anchoring of dynein by Num1 produces defects in respiratory growth. The defective respiratory growth is not causes by lack of functional anchoring of Dynein, but rather by a toxic effect of the misplaced protein, as it can be suppressed by deletion of Dyn1. They arrive at this conclusion by analyzing the effect on a number of separation-of function mutants of Num1 and artificial tethers of the mitochondria-cortex contact site. The phenotype that they report is interesting as it adds a new layer to the function of Num1, suggesting that the anchoring of Dynein at the cortex is not only important for its role in nuclear migration but also for preventing a specific toxicity of the protein for respiratory growth. I think the study would represent a significant advance in the field and be thus suitable for publication, only if the authors show some insight into how the mislocalization of Dynein affects mitochondrial function and respiratory growth.*

We thank the reviewer for providing multiple insightful suggestions on how to determine the mechanism by which unanchored dynein impacts mitochondrial function and respiratory growth.

Each suggestion and comment is addressed in detail below.

Comments for the Author:
1) The phenotype reported is interesting, but the study lacks any mechanistic insight into why lack of Dynein anchoring results in lack of respiratory growth. The authors discuss a few possibilities, including an effect on stability of microtubules or a direct interaction of the protein with mitochondria, but no experiments are made to explore these possibilities. In my opinion, the article would only represent a significant advance in the field that would warrant publication if they provide some insight into why does Dynein mislocalization cause mitochondrial dysfunction. Some ideas on how this could be approached:

a) What about the other components of dynein? Dyn3 does not localize to the cortex but is required for Dyn1 localization at the cortex (Lee et al, JCB 2005). Would deletion of Dyn3 result in the same phenotype regarding mitochondrial function as deletion of Num1?

The initial observation that Dyn3 is required for Dyn1 offloading at the cell cortex made in Lee et al, JCB 2005 has since been corrected. In Lee et al, it was thought that Dyn1 was absent from the cortex but associated with the plus ends of astral MTs. However, in Markus and Lee, BioArchitecture 2011, this interpretation of the data was demonstrated to be incorrect; in the absence of Dyn3, Dyn1 forms aggregates that are not MT associated. Markus and Lee go on to present data that indicate Dyn3 promotes a stable and proper conformation of Dyn1 and that the effects of Dyn3 on Dyn1 localization are likely due to its effects on Dyn1 conformation.

We have included Δ*dyn3* cells in our growth analyses and find the subtle growth defect of Δ*dyn3* cells is similar to that of Δ*dyn1* cells (Fig. 4A). In addition, similar to Δ*num1* Δ*dyn1* cells, Δ*num1* Δ*dyn3 cells ex*hibited a notable increase in the ability to grow in respiratory growth conditions compared to *Δnum1* cells (Fig. S5D). As Dyn3 is required for the stable and proper conformation of Dyn1 and thus activity of dynein, these results suggest that the respiratory growth defect

observed when dynein is unanchored requires functional complexes of dynein.

b) Would overexpression of Dyn1 have a similar effect? (by surpassing the available Num1 binding sites)

We overexpressed *DYN1-GFP*, the dynein heavy chain, and *PAC11-GFP*, the dynein intermediate chain, independently and in combination from both the GPD and TEF promoters (overexpression was confirmed by imaging) and observed no respiratory growth defects. These results suggest that overexpression of individual dynein subunits is not sufficient to cause defects in respiratory growth. It is likely all subunits of the dynein complex would need to be overexpressed at the correct stoichiometry to assemble functional dynein motors. These results lend further support to the idea that the respiratory growth defect observed when dynein is unanchored requires functional complexes of dynein. These data are included in Rebuttal Figure 1A and B.
[NOTE: We have removed a figure which was provided for the referees in confidence.]

c) Is Dynein binding to microtubules required for the toxicity? Would deleting proteins required for delivering Dynein to the plus end rescue the toxicity? (bik1-CTΔ40, see Sheeman et al, Current Biology, 2003)

We have examined the respiratory growth of Δ*num1* cells that express mutant versions of Dyn1 and have included this data in Figure 5F.

More specifically, the following Dyn1 mutants were expressed from the endogenous *DYN1* locus as FLAG-tagged fusions in Δ*num1* cells:

- Dyn1Tail (amino acids 1-1363) - lacks the motor domain and does not depend on plus- end targeting for association with Num1 at the cell cortex (Markus et al., Current Biology 2009)
- Dyn1Motor (amino acids 1364-4092) - non-motile, localizes to the spindle pole body and plus ends of microtubules but is absent from the cell cortex (Markus et al., Current Biology 2009)
- Dyn1ΔMTBD - lacks the microtubule binding domain, is targeted to the plus-ends of microtubules, and exhibits changes in astral microtubule length and dynamics that are equivalent to those seen in Δ*dyn1* cells (Lammers and Markus, JCB 2015; Estrem et al., JCB 2017)

It is important to note that while each Dyn1 mutant behaves differently in terms of localization, none of the mutants form a fully functional dynein complex that is capable of supporting dynein-mediated nuclear migration.

Δ*num1* cells expressing Dyn1Tail, Dyn1Motor, or Dyn1ΔMTBD as the sole source of Dyn1 exhibited respiratory growth similar to Δ*num1* Δ*dyn1* cells. These data, in combination with data demonstrating that all subunits of dynein are required for the respiratory growth defect of Δ*num1* cells, indicate that the full dynein complex and its ability to interact directly with microtubules is required for the respiratory growth observed when dynein is unanchored.

We have attempted experiments in strains lacking Bik1 and in strains expressing Bik1-CTΔ40, which lacks the cargo binding domain of Bik1. Unfortunately, growth defects of the single mutants themselves in combination with variability in growth between clones for *num1 bik1* double mutants have hindered our ability to make any conclusions about the role of Bik1-dependent targeting of dynein to the plus ends of microtubules.

d) Does Dyn1 interact with mitochondria under these conditions? Options to do this would be fluorescence microscopy, or if this is hard to achieve as the authors state in the discussion, alternatives would be biochemical assays:

- *Co-purification of Dynein with mitochondria under these conditions.*
- *Pull down experiments to detect the binding partner on mitochondriao BioID to detect the molecular microenvironment.*

We have examined the association of dynein with mitochondria using cell biological and biochemical techniques and find no evidence of a mitochondria-dynein association. Specifically, using confocal microscopy and a high-sensitivity HyD (GaAsP) detector, we visualized Dyn1- 3xGFP

in WT cells and cells lacking Num1. Within the limits of detection, we do not see any appreciable co-localization between Dyn1-3xGFP and mitochondria. In addition, we have isolated mitochondria from WT and Δ*num1* cells expressing Pac11-yEGFP. Dyn1 is a 471 kDa protein and is difficult to visualize on western blots. Therefore, we tracked Pac11, the dynein intermediate chain, as a proxy for the multisubunit dynein complex. The localization of Pac11 follows that of Dyn1. Similar to the imaging data, we find no appreciable association of Pac11 with mitochondria by biochemical fractionation. Thus, by these methods, we are not able to conclude that there is any association between dynein and mitochondria in either WT or Δ*num1* cells.

The pull-down and BioID experiments are excellent suggestions to determine how the interaction network of dynein differs in the absence of Num1 or dynactin components. However, we feel these experiments are beyond the scope of the current manuscript. First, BioID or TurboID is not trivial to do in yeast due the high concentration of biotin in cells. If proteins are expressed at high levels, the levels of endogenous biotin are less problematic. However, Dyn1 is not a highly abundant yeast protein. Second, if promising targets are identified using BioID/TurboID or IP- mass spectrometry, the follow up studies on these candidates will likely yield promising lines of investigation that will extend beyond the scope of the present manuscript.

e) Could they perform a screen to find mutants of Dyn1 which are not toxic under these conditions?

We appreciate the reviewer's suggestion. As detailed above (please see response to Reviewer 1 Comment 1c), we have analyzed various mutants, including those suggested by the reviewer, to disrupt various aspects of dynein function in a candidate screening approach. While an unbiased screen has the potential to uncover novel dynein mutants, we feel the candidates we analyzed specifically disrupt a number of specific properties and activities of dynein. Based on the analysis of these mutants, we can conclude that the full dynein complex and its ability to interact directly with microtubules is required for the respiratory growth observed when dynein is unanchored.

2) They show many assays in which they quantify colony size and many assays where they quantify the fraction of the cell cortex containing mitochondria. In these assays they show data for individual colonies/cells, from at least 3 independent experiments according to Figure legends. However, the data is shown as just one set. They should separate the data from each experiment in some way (either side by side or color coding), and show the average of each experiment. In this way, the figures would not only depict the biological variability of the system but also the reproducibility. In addition, statistical comparisons should be shown, to see if the observed differences are significant. Importantly, the statistical comparisons should be done using the averages of the experiments, and not all the single cell/colony data. More information on how to depict and perform statistical comparisons on such data is discussed in Lord, Velle, Mullins and Fritz-Laylin, JCB, 2020

We thank the reviewer for these very useful suggestions. For the colony size assays, we now depict the means from each individual experiment for a given genotype as circles in addition to the grand mean of the combined experiments. The means of the independent experiments for each genotype were used for statistical comparisons as described in Lord et al., JCB 2020.

3) I would suggest a title that states the finding of the paper in a more straightforward manner.

The reviewer raises a good point. We have changed the title of the manuscript to “Loss of Num1-mediated cortical dynein anchoring negatively impacts respiratory growth,” which more directly conveys our findings.

Reviewer 2

Advance Summary and Potential Significance to Field: *This study examines the role of the multifunctional mitochondrial tether Num1 in mitochondrial spatial organization and cell growth. Num1 is characterized as a mitochondrial-PM tether, but also interacts with the cytoskeleton to control organelle organization and inheritance. This study investigates the relative importance of each of these Num1 interactions and how they relate to yeast growth and respiration. Through elegant structure-function analysis, Num1 targeting to the PM and its associated mitochondrial*

polarization to the cell surface is found to influence yeast growth and respiration. Loss of Num1 leads to reduced cell growth and respiration, but this can be rescued through artificial Num1 fragments that target to the eisosomes on the PM. However, all artificial tethers required the Num1 CC domain, suggesting another role for the CC domain other than simply mitochondrial tethering was required for Num1 function. Surprisingly experiments suggest that the CC domain's role in positioning the dynein complex at the cell surface, which aids in yeast nuclear inheritance during budding, is the key role for Num1. In support of this, artificially delivering Dyn1 to the PM via an artificial anti-GFP nanobody strategy can rescue growth defects in Num1KO. The study indicates that the growth phenotype observed in Num1-deleted yeast is primarily due to mislocalized dynein rather than defective mitochondrial-PM contact.

Comments for the Author: *The study appears rigourous and the experiments are well controlled and interpreted. The model, that loss of Num1 mitochondrial tethering is not the causative effect of the Num1 growth defect, is surprising. The alternative model, that Dyn1 mislocalization is the true culprit, is well investigated, although somewhat underexplored at the end of the study. The only significant concern would be that the study does not offer a more direct explanation for why mis-localized Dyn1 causes defects in growth. Following up on this with a few characterizing experiments would enhance the already thorough study.*

We thank the reviewer for their positive and insightful comments.

1) The study indicates that the mislocalization of Dyn1, rather than Num1 mito-PM tethering or nuclear inheritance, is causative of the growth and respiration defects observed in Num1 KO. What issue does having mislocalized Dyn1 have on yeast and mitochondrial health? Since the Dyn1 is likely remaining on microtubules, does this effect microtubule dynamics or other trafficking events?

We have tried to gain insight into the mechanism by which mislocalized dynein leads to defects in respiratory growth (please see responses to Reviewer 1 Comments 1a-e). We have conducted additional experiments that indicate the full dynein complex and its ability to interact directly with microtubules are required for the defect in respiratory growth observed in cells lacking Num1. We feel the additional work required to gain further understanding of the mechanism in question will take significant time to complete and is beyond the scope of the current manuscript.

2) Similar to the point above, how does Num1 loss impact Dyn1 abundance? Can manipulating Dyn1 levels through AID or over-expression also bypass Num1 loss?

Please see the response to Reviewer 1 Comment 1b regarding overexpression of Dyn1 and Pac11.

For the manipulation of dynein levels/activity through auxin inducible degradation (AID), we first tagged Dyn1 or Pac11, the dynein intermediate chain, with an AID tag in cells lacking Kar9, as a control for loss of dynein activity. When grown in the presence of auxin, both Δ*kar9 DYN1-AID* or Δ*kar9 PAC11-AID* cells exhibited a growth defect. While the growth defect was more severe for Δ*kar9 PAC11-AID* cells than for Δ*kar9 DYN1-AID* cells (included in Rebuttal Fig. C and D), neither strain exhibited a defect as severe as what is typically seen for Δ*kar9* Δ*dyn1* cells. These results indicate that neither Dyn1-AID nor Pac11-AID are being fully depleted in the presence of auxin.

We examined how the depletion of Dyn1-AID and Pac11-AID affects the respiratory growth of Δ*num1* cells. We found that Δ*num1 DYN1-AID* and Δ*num1 PAC11-AID* cells grown in the presence of auxin grew better than those grown in the absence of auxin (+DMSO) and also better than Δ*num1* cells (included in Rebuttal Fig. C and D). These results are consistent with the finding that dynein is required for the growth defect observed in cells lacking Num1. Because the depletion of Dyn1-AID and Pac11-AID in the presence of auxin is incomplete, we have decided to not include these data in the manuscript. We would be happy to include the data should it be deemed necessary.[NOTE: We have removed a figure which was provided for the referees in confidence.]

3) There is a general lack of statistical analysis for some of the panels. For example Fig 3D, Fig 4A. Please provide statistical tests and information.

We have included statistical analyses for all quantified data and descriptions of the analyses in the methods.

Reviewer 3

Advance Summary and Potential Significance to Field: *The manuscript by White et al dissected the role of Num1 in mitochondrial homeostasis. This is complex since Num1 is has two main functions: the first in tethering the Plasma membrane-mitochondria-ER three-way contact site and the second in nuclear inheritance to the bud. The authors found that the CC domain of Num1 is the one required for mitochondrial function. Using synthetic tethering and growth assays they nicely showed that the effect on mitochondria was not due to either reduction in contact site or defects in nuclear inheritance. Rather, and quite surprisingly, it is the misregulation of cortical dynein anchoring that is important for proper mitochondrial function. This study is a beautiful example of how mechanistic dissection of a biological process should be performed and is well-executed and quantified. Most importantly, it broadens our knowledge regarding the multifunctional Num1 protein, dynein regulation and mitochondrial function and is therefore highly worthy of publication in JCS following consideration of the comments stated below:*

We thank the reviewer for their positive and supportive review.

Comments for the Author:

Textual:

1) Line 155: ".. crucial functions of mitochondria is the production of energy …". Please rephrase the sentence since mitochondria do not produce energy (a physical impossibility), but rather produce ATP molecules.

We have changed the text as follows: crucial functions of mitochondria is the production of ~~energy~~ ATP

2) We suggest to move Figure 1F to supplementary figures since mdm36 and dyn1 mutations are not the main focus of the paper. There is no view of Num1 mutants in these conditions and is merely a control for the panel coming before it.

We agree with the reviewers suggestion and have moved Figure 1F to the supplementary figures.

3) We suggest to make a new "Results" section with its own header for the text describing the results in Figure 3 (that right now is continuous with the text describing Figure 2). This will help highlight this important section.

We agree with the reviewer's suggestion and have added a new header entitled "Restoring mitochondria-plasma membrane tethering in the absence of Num1 is not sufficient to rescue respiratory growth" for the results that describe the data shown in Figure 3.

4) We suggest to add a schematic illustration of the final mechanism that is described in the discussion section (lines 415-416) as a closing model Figure.

A final model figure has been added to the manuscript.

Experimental:

1) Figure 1C- The figure shows higher expression levels of Num1 in respiratory condition compared to fermentation condition. While the authors show "whole protein" levels in the Supplementary as proof of equal loading - this is not sufficient for quantitation. We suggest addition of a loading control such a housekeeping protein and quantitation of this blot.

We apologize for any confusion that was caused due to our initial description of the quantification and normalization for the western blot shown in Fig. 1C. We have clarified the description of our quantification and normalization method in the Methods; we normalized the Num1 protein signal in each lane to that of the total protein for that lane. An example western blot and its corresponding Revert-stained membrane are shown in Fig. 1C and S3A. Total protein normalization has become the recommended method for western blot quantifications (Pillai-Kastoori et al., Analytical Biochemistry 2020). Many journals, including the Journal of Biological Chemistry (PMID: 26657753), strongly recommend using total protein stains instead of housekeeping proteins as a loading control.

2) Figure 1D - The fermentation condition are confusing since they were used to normalize everything to so, of course, the fold change is 1. We suggest to remove this bar and leave only the respiration.

We have modified the graph as suggested by the reviewer.

3) Loss of Mdm36 is suggested not to have any effect on growth (Figure 1). However, Mdm36 is a hypomorph of Num1 so it is clear that it will have less effect. The temperature that was used in this experiment was 35OC, however, in this condition Num1 shows little effect. Therefore, to really state this the growth rate of Δmdm36 strain should be measured in 37OC – conditions in which loss of Num1 had a dramatic effect.

We appreciate the reviewer's concern. We have included spot test growth assays that compare the growth of Δ*mdm36* cells to WT and Δ*num1* cells at 30, 35, and 37ºC. Even at 37ºC, the respiratory growth of Δ*mdm36* cells is similar to that of WT cells. These data are now included in Fig. S2B.

4) Figure 2B- Two important controls are missing- Full length Num1 tagged with GFP on its C terminus to check that the GFP tagging is not the cause of the growth defect in respiratory condition since all the different strains were tagged with GFP. Another important control is tagging only the PH domain of Num1 with GFP. It is possible to tag one of the mitochondrial membrane proteins such as Tom20/Tom70 with aGFP and observe if this synthetic tethering can rescue the phenotype.

We have verified that the addition of GFP to full length Num1 in an otherwise WT background does not affect respiratory growth (see Fig. 2B, Num1-GFP).

We have also included growth assay data for strains in which the PH domain of Num1 fused to the αGFP nanobody (PH-αGFP) is used instead of Pil1-αGFP to recruit Num1ΔPH-GFP, GFP- Mdv1NTE or Tom70TM-GFP to the plasma membrane and restore mitochondria-plasma membrane tethering. We find that when Num1 is reconstituted using the GFP-αGFP system (i.e. cells expressing Num1ΔPH-GFP and PH-αGFP), respiratory growth is rescued. In contrast, respiratory growth is not rescued when mitochondria are synthetically tethered to the plasma membrane in cells expressing PH-αGFP along with GFP-Mdv1NTE or Tom70TM-GFP. These results are included in Fig. S4B and C and provide further evidence that the presence of the Num1 CC domain in a mitochondria-PM tether construct, and not the Num1 PH domain, is required to rescue the respiratory growth of *num1* cells.

5) All graphs presented in this paper should show the P values or other statistical significance measurement between the different strains compared.

We have included statistical analyses for all quantified data.

Second decision letter

MS ID#: JOCES/2022/259980

MS TITLE: Loss of Num1-mediated cortical dynein anchoring negatively impacts respiratory growth

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.