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Overexpression of Mdm36 reveals Num1 foci that mediate dynein-dependent microtubule sliding in budding yeast

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MS TITLE: Overexpression of Mdm36 reveals Num1 foci that mediate dynein-dependent microtubule sliding in budding yeast

AUTHORS: Safia Omer, Katia Brock, John Beckford, and Wei-Lih Lee
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.)

All three reviewers found the study to be interesting, thorough and well executed. As you will see, the reviewers raise few concerns 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. In particular, reviewer 1 has raised concern about the ratiometric approach to measure number of fluorescent molecules and has suggested another approach to more accurately quantify this data. Both reviewer 1 and 3 have raised concern about the overexpression approach used for the study. For instance, if two distinct populations of Num1 do exist (one anchoring dyneins and the other mitochondria), this should be apparent even in a WT background. Additionally, reviewer 1 (pt# 4 &5) has raised concern about the conflicting data in previous studies on spindle positioning defect in mdm knockout. Reviewer 2 has raised an important concern (pt#2 and 3) regarding the mechanism of clustering of the Num1 mutant used in the study.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

In their manuscript entitled "Overexpression of Mdm36 reveals Num1 foci that mediate dynein-dependent microtubule sliding in budding yeast", Omer et al. demonstrate that a small number of dyneins is sufficient for proper positioning the mitotic spindle. Large Num1 clusters, that are present in Mdm36 overexpressing cells, are seemingly devoid of dynein but instead tether mitochondria. Therefore, the authors propose that distinct populations of Num1 serve to tether mitochondria and dynein respectively, with the latter preferring to localize on smaller clusters. The experiments that have been performed are careful and thorough. However, the manuscript contains some major discrepancies with prior literature in the field, that need to be addressed prior to publication.

Comments for the author

1. The ratiometric approach employed in this work to determine Num1 number per spot has been shown to be inaccurate, with the number of Cse4-GFP in each anaphase cluster ranging from 80-122. Given that this is the case, the authors should refrain from making any claims on the number of Num1 molecules in any cluster, unless the numbers are corroborated by photobleaching experiments. The photobleaching steps of Num1-GFP that would be apparent in these experiments will provide a good estimate of the number of molecules in each spot. In the absence of photobleaching experiments, the authors should only make comparisons of intensities of foci (and not numbers in these foci).
2. In Fig. 1A, the contrast of the images has to be kept constant between WT Mdm36^{ox} and Mdm36Δ cells so as to be able to see fainter foci (if any) in the bud of Mdm36^{ox} cells.
3. Likewise, in Fig. 1B, the WT intensity profile should be normalized to that of Mdm36^{ox} to check if fainter foci (with intensities similar to some of those in WT cells) exist in the Mdm36^{ox} buds.
4. Based on their results, the authors conclude that ~3 dynein molecules (anchored by ~6 Num1 molecules in a cluster) are sufficient for spindle pulling. The authors also show that Mdm36Δ cells exhibit ~13 Num1 molecules per cluster, and therefore dynein-driven spindle positioning would presumably intact in these cells. However, Kraft et al. 2017 have shown a spindle positioning defect in Mdm36Δ cells, indicating abrogation of dynein activity. How do the authors resolve this discrepancy?
5. The authors' results are in contradiction to Kraft et al. 2017 and Schmit et al. 2018, where it was demonstrated that Num1 cluster formation is necessarily driven by mitochondria and that these clusters serve as sites for dynein anchoring. Kraft et al. 2017 additionally show colocalization of dynein and mitochondria on the same Num1 foci, and a spindle positioning defect in their mito^{ts} mutant at 37°C. Could the authors discuss why their results are different? One step in trying to resolve this issue could be 2-color imaging of mitochondria and dynein to visualize colocalization (if any) of the two components in Mdm36^{ox} mutants.
6. The Introduction and Discussion sections would benefit from a more comprehensive overview of fission yeast, where some of the questions asked by the authors in this work (such as factors influencing cluster assembly/size, and whether dynein and mitochondria occupy the same clusters) have been addressed. Specifically, PMIDs 28292899, 30649994, 31582398 provide examples of what should be covered.
7. Line 18: In the abstract, please include a definition of Mdm36 where it is mentioned for the first time to aid comprehension by non-experts in the field.

Reviewer 2

Advance summary and potential significance to field

Num1 is a cell cortex associated protein with a dual role in mitotic spindle positioning and mitochondrial distribution during cell division in budding yeast. Omer et al generated two morphologically and functionally distinct forms of Num1 clusters by overexpression of its assembly factor Mdm36. They propose a model in which small, Mdm36 independent clusters function in dynein anchoring and spindle pulling, whereas large, Mdm36 dependent clusters exclude dynein binding and function in mitochondrial tethering. This is an interesting and well executed study.

Comments for the author

The following points should be considered by the authors.
Major points
1. Fig. 1E shows co-fractionation of Num1-GFP with an ER marker protein in sucrose density gradient centrifugation. As Num1 is known to interact with mitochondria it would be important to include a mitochondrial marker in this experiment.
2. The authors tested whether interactions with mitochondria are required for the formation of two morphologically and functionally distinct Num1 patches in Mdm36-ox cells. They introduced mutations into Num1 that were previously shown to disrupt interactions with mitochondria (Num1-3E). To estimate the effect of the 3E mutations on Num1 clustering, they should directly compare in the same experiment Num1-3E/Mdm36-ox with Num1/Mdm36-ox. Unfortunately, Num1/Mdm36-ox is missing in Fig. 5.
3. If mitochondrial interactions are required for Num1 patch formation, and if these interactions are blocked in Num1-3E mutants, it would be expected that the block of mitochondrial interactions also block the formation of large Num1-3E clusters in Mdm36-ox cells, which is obviously not the case. How can this discrepancy be explained? Also, it should be explained how mitochondrial morphology of Num1-3E mutants could be restored upon Mdm36 overexpression if this Num1 variant is unable to interact with mitochondria.
4. Chacko et al. (JCB 2019, 218:3560-71) recently analyzed the role of Mcp5, the Num1 homolog in S. pombe, in tethering of mitochondria during meiosis. They found that the presence of dynein on a Mcp5 cluster precluded the attachment of mitochondria to the same cluster. This work should be discussed and cited.
Minor points 5. It is known that Num1 is asymmetrically distributed between mother and bud in wild type cells, and that it appears in the bud very late in the cell cycle. The authors show that overexpression of Mdm36 leads to the assembly of large Num1 clusters specifically in the mother cell. Do these bright Num1 foci also appear in the bud late in the cell cycle, i.e. in very large buds of Mdm36-ox cells?
6. Micrographs should be displayed much larger in Fig. S1B.
7. The authors mention that they observed many Mdm36-ox cells with persistent mitochondrial tether points. It would be nice if they could show representative examples of their videos.

Reviewer 3

Advance summary and potential significance to field

The main take away message from this paper is the evidence that there are 2 subpopulations of Num1 having distinct roles in spindle pulling and mitochondrial tethering, and that there is s dynein-dependent microtubule sliding at Num1 foci. Formation of larger Num1 clusters are important for mitochondrial tethering but not for spindle orientation.

This corrects other published findings suggesting that larger Num1 clusters are needed for spindle pulling.

Comments for the author

Overexpression of Mdm36 reveals Num1 foci that mediate dynein-decedent microtubule sliding in budding yeast

Omer at al. analyse in this manuscript how clustering of the cortical dynein binding protein Num1 affects its function in spindle positioning and mitochondrial-anchoring. The authors start with the overexpression of MDM36 that leads to a strong enhancement of cortical Num1 patches and their clustering at the mother cortex. Using the CENP-A homologue Cse4-GFP as standard for the signal intensity of cellular GFP molecules, they counted in WT cells 31 Num1-GFP per cortical patch. This number was increased to 137 when MDM36 was overexpressed. Surprisingly, clustering of Num1 patches upon MDM36 over-expression in the mother cell compartment did not affect the dynein spindle orientation pathway. This finding was confirmed by testing genetic interactions of MDM36 over-expression and kar9Δ and the frequency of spindle crossing through the bud neck. The authors next asked whether dynein localization is affected by MDM36 over-expression. The number of cortical dynein patches was reduced. Interestingly, the Dyn1-3GFP intensity of patches was reduced upon MDM36 overexpression. While the signal intensity at microtubule plus ends was increased. From this the authors conclude that the off loading mechanism of dynein from the microtubule ends is defective. They further show that dynein at the bud cortex is able to capture microtubule plus ends and to pull microtubules by a sliding mechanism even when most Num1 was clustered in the mother compartment because of MDM36 over-expression. Next, they analysed whether enhancement of Num1 patches affect mitochondria. Indeed, they observed a stronger tethering phenotype upon MDM36 over-expression. They report division of labour between bright Num1 patches and dim patches, the first being involved in mitochondria tethering. Finally, the authors use a Num13E mutant that lacks the ability to interact with mitochondria but still is accessible for clustering by Mdm36. With this mutant in hand, they address the question whether mitochondria interaction of Num1 is important for patch assembly in the bud as reported recently. In this study by Omar et al., however, the authors observe that Num13E did not affect spindle orientation regardless of MDM36 overexpression. num13E was also not synthetically lethal with kar9Δ.
In summary, this is an interesting manuscript on the role of Num1 clusters and dynein in spindle orientation and tethering of mitochondria. It convincingly shows that clustering of Num1 is important for mitochondria tethering but not for pulling of the anaphase spindle. The only slight criticism is that all experiments are based on MDM36 over-expression. Num1 clustering can be introduced in other ways. Overall, the manuscript is of high technical quality. Taken this together, I recommend publishing of this manuscript in JCS after minor revision.

Minor points

1. The introduction provides a good, generalized background of the topic. However, the aim of the study is mentioned as, “we set out to characterize the role of Mdm36 in Num1 clustering”. This is not correct.
Mdm36 is used as a tool to cluster Num1.
2. The authors could provide more background on how specific experiments were done in order to keep the reader who is not working in this field on board. The GFP counting in Fig. 1 is an example.
3. Does num1LL bind dynein when MDM36 is overexpressed? If the authors have the data, it would be good to add to the manuscript.
4. The authors describe in results that off loading of dynein from microtubule plus ends is affected upon MDM36 over-expression. It might be worth to add one or two sentences in the Discussion about this potential mechanism.

First revision

Author response to reviewers' comments

We thank the reviewers for valuable comments and suggestions. We have included below: (1) a list outlining the new experiments added to the manuscript, (2) a list summarizing the changes made to the figures and videos, and (3) a detailed point-by-point response to each reviewer’s comments.

New experimental data added to the manuscript

1. In response to reviewer #1 (point #1), we have verified the absolute copy number of molecules in Num1-GFP foci using two different approaches. First, we performed new ratio measurements using a different fluorescence standard, Mif2-GFP. Second, as requested, we performed stepwise photobleaching experiments to corroborate the numbers determined based on using the Cse4-GFP standard. These results are described in the revised text (page 7, lines 119-130) and presented in the new panels Figure S2A-C.

2. In response to reviewer #1 (point #4), we have investigated dynein pathway function in *mdm36Δ* cells. We found that *MDM36* deletion did not affect dynein function and localization in our YEF473A strain background. We show that loss of Mdm36 did not result in a misoriented anaphase spindle phenotype (new Figure 5F), nor did it affect the frequency and distance of preanaphase spindle movements in a spindle crossing assay (new Figure 5G and H). We also show that dynein targeting to the astral MT plus ends and the cell cortex was not affected in *mdm36Δ* compared to WT cells (new Figure 5I and J). These data lend further support to our model that dim patches with a small ensemble of Num1 molecules (like those observed in *mdm36Δ* cells) are sufficient for dynein-driven spindle positioning function.

3. As suggested by reviewer #1 (point #5), we have performed 2-color time-lapse imaging of mitochondria and dynein to investigate whether these two components colocalize in Mdm36^{OX} cells (new Figure 3E). The results show that cortical Dyn1-3GFP foci can be clearly observed without being associated with cortically-tethered Cox4-mCherry-labeled mitochondria, supporting the idea that dynein and mitochondria do not bind to the same Num1 foci.

4. As requested by reviewer #2 (point #1), we have performed sucrose gradient sedimentation analysis with a mitochondria marker (see new Figure 1E). The results show that mitochondria co- sedimented with the fractions containing ER and Num1 in both Mdm36^{OX} and WT cell lysates.

5. As requested by reviewer #2 (point #2), we have included quantification of WT Num1-GFP patch intensity in Mdm36^{OX} cells to determine the effect of the 3E mutations on Num1 clustering (see new Figure 5A and B). The results show that, although both Num1^{3E}-GFP and Num1-GFP patches were dramatically enhanced upon Mdm36 overexpression, the levels of the enhanced Num1^{3E}-GFP patches were significantly lower compared to those of Num1-GFP patches in Mdm36^{OX}, indicating that Num1's interaction with mitochondria is indeed required for proper Num1 clustering.

6. In response to reviewer #3 (point #3), we have examined cortical dynein localization in *num1^{LL}* cells overexpressing Mdm36 (new Video 2). The results show that cortical dynein localization was abolished by *num1^{LL}* mutations (0 out of 288 *num1^{LL}* Mdm36^{OX} cells exhibited stationary cortical Dyn1-3GFP foci), indicating that *num1^{LL}* does not bind dynein when *MDM36* is overexpressed.

Changes to the Figures and Videos

Figure 1 Panel A is replaced with unadjusted, raw images of Num1 patches (as requested by reviewer #1). Panels B-D are unchanged. Panel E contains new western blot showing sedimentation profile of the mitochondrial marker Por1 (as requested by reviewer #2).

Figure 2 Panel A is re-plotted to show WT intensity profile normalized to that of Mdm36^{OX} (as requested by reviewer #1). Panels B-F are unchanged.

Figure 3 Panels A-D are unchanged. Panel E is new data showing the lack of colocalization between cortical dynein and mitochondria in Mdm36^{OX} cells (as requested by reviewer #1).

Figure 4 This figure is unchanged.

Figure 5	Panels A-B contain new image and quantification for Num1-GFP patches in *MDM36*^{OX} cells (as requested by reviewer #2). Panels C-E are unchanged. Panels F-J are new data showing quantification of dynein function and localization in *mdm36Δ* cells.
Figure 6	Panels A and B are the old Figure 5F and G, respectively.
Figure S1	Panel A is unchanged. Panel B is enlarged (as requested by reviewer #2).
Figure S2	Panels A-C are new data showing number of molecules per patch measured by using the Mif2-GFP standard and by stepwise photobleaching method (as requested by reviewer #1). Panel D is old Figure S1C.
Figure S3	This figure is the old Figure S2.
Video 1	This movie has not been changed.
Video 2	This is new data showing Dyn1-3GFP localization in *num1*^{LL} *MDM36*^{OX} cells.
Video 3	This is the old Video 2.
Video 4	This is the old Video 3.
Video 5	This is the old Video 4.
Video 6	This is a new video showing persistent mitochondrial tethering in Mdm36^{OX} cells (as requested by reviewer #2).
Video 7	This is the old Video 5.
Video 8	This is the old Video 6.

Detailed Point-by-Point Response to Reviewer #1:

We thank the reviewer for valuable comments and suggestions that help improve our manuscript.

1. ***The ratiometric approach employed in this work to determine Num1 number per spot has been shown to be inaccurate, with the number of Cse4-GFP in each anaphase cluster ranging from 80-122. Given that this is the case, the authors should refrain from making any claims on the number of Num1 molecules in any cluster, unless the numbers are corroborated by photobleaching experiments. The photobleaching steps of Num1-GFP that would be apparent in these experiments will provide a good estimate of the number of molecules in each spot. In the absence of photobleaching experiments, the authors should only make comparisons of intensities of foci (and not numbers in these foci).***

We have now used two different methods to corroborate the number of molecules determined based on the Cse4-GFP standard. First, we performed new ratio measurements using a different fluorescence standard, Mif2-GFP, which contains 58 molecules per cluster (see Fig. 19.2 in Verdaasdonk et al. Methods Cell Biol 2014, PMID 24974037). The results show that, for all three yeast strains, the absolute copy numbers of Num1 per patch are very similar to the values determined using the Cse4-GFP standard (compare Fig. 1C with new Fig. S2A; using Cse4-GFP standard: 31, 137, 13 for WT, Mdm36^{OX}, and *mdm36Δ* strains; using the new Mif2-GFP standard: 38, 139, and 15 for the same yeast strains, respectively). Second, as suggested, we have performed stepwise photobleaching experiments on WT Num1-GFP cells to measure the intensity loss during bleaching using the STEPFINDER software (see new Fig. S2B and S2C), as described by Thankachan et al. PNAS 2017 (PMID 28292899). The results confirm that the values obtained by ratio measurements based on the Cse4-GFP standard are indeed reliable (36 versus 31 copies of Num1-GFP for WT foci measured by the photobleaching approach and the Cse4-GFP ratiometric approach, respectively). We thank the reviewer for the push to get these data, which is now described in the revised text (page 7, lines 119- 130).

2. *In Fig. 1A, the contrast of the images has to be kept constant between WT, Mdm36^{ox} and Mdm36Δ cells so as to be able to see fainter foci (if any) in the bud of Mdm36^{ox} cells.*

We would like to clarify that the images for all three strains in the original Fig. 1A were displayed with the same contrast adjustment, after they were acquired under the same conditions with the same imaging settings. Nonetheless, to ensure that dim foci in the bud of Mdm36^{OX} cells can be clearly visualized, we have now replaced the images for all three strains with raw images that have not been contrast adjusted. For clarity, we have also added arrows to indicate the dim foci seen in the bud of Mdm36^{OX} cells (see new Fig. 1A).

3. *Likewise, in Fig. 1B, the WT intensity profile should be normalized to that of Mdm36^{OX} to check if fainter foci (with intensities similar to some of those in WT cells) exist in the Mdm36^{ox} buds.*

As requested, we have now normalized the WT intensity profile in Fig. 2A (referenced above accidentally as Fig. 1B) to that of Mdm36^{OX}. The results show that dim foci exist in the bud of both WT and Mdm36^{OX} profiles (indicated by arrows in the new Fig. 2A). Thanks for the suggestion.

4. *Based on their results, the authors conclude that ~3 dynein molecules (anchored by ~6 Num1 molecules in a cluster) are sufficient for spindle pulling. The authors also show that Mdm36Δ cells exhibit ~13 Num1 molecules per cluster, and therefore dynein-driven spindle positioning would presumably intact in these cells. However, Kraft et al. 2017 have shown a spindle positioning defect in Mdm36Δ cells, indicating abrogation of dynein activity. How do the authors resolve this discrepancy?*

We have addressed this issue by first investigating whether dynein pathway function is indeed intact in *mdm36Δ* cells in our hands. We performed dynein functional assays and examined dynein localization in cells lacking Mdm36. We discovered that deletion of *MDM36* did not result in a misoriented anaphase spindle phenotype (new Figure 5F). We also found that the frequency and the distance of preanaphase spindle movements observed for a *mdm36Δ kar9Δ* strain in a spindle crossing assay are statistically indistinguishable to those of a *kar9Δ* strain (new Figure 5G and H). Furthermore, dynein targeting to the astral MT plus ends and the cell cortex is not affected in *mdm36Δ* relative to WT cells (new Figure 5I and J). These results *not only* confirm that dynein pathway function is intact in *mdm36Δ* cells *but also* lend further support to our model that dim patches with a small ensemble of Num1 molecules are sufficient for dynein anchorage and spindle positioning function.
However, as indicated by the reviewer above, our data appear to be in conflict with a result in Kraft and Lackner (JCB 2017) showing that *mdm36Δ* cells exhibited an elevated level of misoriented spindles. We would like to point out that Kraft and Lackner (JCB 2017) also showed that the fold increase in the level of the misoriented spindle phenotype for *mdm36Δ* (relative to WT) appears to be highly dependent on the strain background: ~8-fold increase in the BY4741 background, whereas only ~2-fold increase in the W303 background (based on measurements taken directly from the graphs in Figure 4B and 4E of Kraft and Lackner, JCB 2017). Thus, we speculate that the discrepancy between our results and theirs might be due to a difference in the genetic background, since our *mdm36Δ* mutant was generated in a different background (i.e., YEF473A). As described above, we have now performed additional experiments (i.e., anaphase spindle orientation assay, spindle crossing assay, and dynein localization assay) to confirm that *MDM36* deletion does not affect dynein activity in our strain background. We thank the reviewer for the push to strengthen our data, which is now described in the revised text (page 16-17, lines 360-387).

5. *The authors' results are in contradiction to Kraft et al. 2017 and Schmit et al. 2018, where it was demonstrated that Num1 cluster formation is necessarily driven by mitochondria and that these clusters serve as sites for dynein anchoring. Kraft et al. 2017 additionally show colocalization of dynein and mitochondria on the same Num1 foci, and a spindle positioning defect in their mito^{ts} mutant at 37° C. Could the authors discuss why their results are different? One step in trying to resolve this issue could be 2-color imaging of mitochondria and dynein to visualize colocalization (if any) of the two components in*

Mdm36^{ox} mutants.

To clarify, our results are not in complete contradiction to Kraft and Lackner (JCB 2017) and Schmit et al (Cell Cycle 2018). Our analysis of Num1^{3E} mutant shows that Num1's interaction with mitochondria is required for the formation of large Num1 clusters at the cell cortex, in agreement with the notion that mitochondria drive the assembly of cortical Num1 patches, as proposed by Kraft and Lackner (JCB 2017). However, contrary to Kraft and Lackner, our analysis shows that bright and large Num1 patches that are dependent on mitochondrial interactions and Mdm36 are dispensable for dynein pathway function. To explain this, we would like to first point out that peripheral cortical ER also plays an important role in the formation of Num1 patches regulating dynein function in spindle positioning in the bud (see Omer et al. eLife 2018 and Chao et al. Cell 2014). In the *mito^{ts}* experiment carried out by Kraft and Lackner, dynein function was assayed in the absence of Ypt11, a protein previously shown to be required for ER segregation into the bud (see Figure 1C and 1D in Swayne et al. Curr Biol 2011 and Figure 8 in Buvelot et al. Mol Cell Biol 2006). Thus, the discrepancy between our observations and those of Kraft and Lackner likely arises from the difference in the levels (or the integrity) of cortical ER underlying the plasma membrane in the strains used to ascertain dynein's function in spindle orientation. In our study, we based our conclusions on cells with intact Ypt11 function, allowing us to avoid artifacts due to defects in cortical ER inheritance and/or formation of ER-dependent Num1 patches in the bud. Additionally, although Kraft and Lackner showed that Dyn1 colocalizes with mitochondria on the same Num1 foci, it remains unclear whether the observed Dyn1, which was found in the mother cell cortex (see Figure 3 in Kraft and Lackner, JCB 2017), is involved in spindle pulling activity. In our work, in addition to showing that Dyn1 differentially localizes to dim cortical Num1 patches (Fig. 3D), we also demonstrate by monitoring astral MT interaction with the cell cortex that dim Num1 patches are directly involved in dynein-mediated spindle pulling activity (i.e., pulling via microtubule sliding mechanism; see Fig. 4B and Video 3). The cortical Dyn1 seen by Kraft and Lackner might represent a pool of inactive dynein, since we rarely observed MT sliding occurring over the brightly enhanced Num1 clusters, which we have shown to be associated with mitochondria. Alternatively, it is possible that a dim Num1 patch with an offloaded dynein might become available for mitochondria binding after the spindle is properly aligned or after the offloaded dynein is inactivated. We would like to point out that our results are in agreement with Chacko et al. (JCB 2019) where it was reported that mitochondria do not bind to preexisting Mcp5-dynein foci in a meiotic *S. pombe* cell undergoing horsetail nuclear movements. Nevertheless, as suggested by the reviewer, we have now performed 2-color imaging of mitochondria and Dyn1 to investigate whether these two components colocalize in Mdm36^{OX} cells. The results show that cortical Dyn1-3GFP foci can be clearly observed without being associated with cortically-tethered Cox4-mCherry-labeled mitochondria. These results are presented in the new panel Fig. 3E.

6. The Introduction and Discussion sections would benefit from a more comprehensive overview of fission yeast, where some of the questions asked by the authors in this work (such as factors influencing cluster assembly/size, and whether dynein and mitochondria occupy the same clusters) have been addressed. Specifically, PMIDs 28292899, 30649994, 31582398 provide examples of what should be covered.

We have now mentioned the fission yeast work and cited them appropriately in the Introduction (page 4, lines 69-73) and Discussion (page 18, lines 406-412) sections. We apologize for the oversight. Thanks for pointing this out to us.

7. Line 18: In the abstract, please include a definition of Mdm36 where it is mentioned for the first time to aid comprehension by non-experts in the field.

We have now added an introduction to Mdm36 where it is first mentioned in the abstract by changing "….is limited by Mdm36" to "….is limited by its assembly factor Mdm36". We thank the reviewer for pointing this out to us.

Detailed Point-by-Point Response to Reviewer #2:

We thank the reviewer for valuable comments and suggestions that help improve our manuscript.

1. *Fig. 1E shows co-fractionation of Num1-GFP with an ER marker protein in sucrose density gradient centrifugation. As Num1 is known to interact with mitochondria it would be important to include a mitochondrial marker in this experiment.*

As requested, we have included the sedimentation profile of mitochondria as determined by immunoblotting against the mitochondrial marker protein Por1. The results show that mitochondria co- sedimented with the fractions containing ER and Num1 in both Mdm36^{OX} and WT cell lysates. These data are presented in the new figure panel Fig. 1E.

2. *The authors tested whether interactions with mitochondria are required for the formation of two morphologically and functionally distinct Num1 patches in Mdm36-ox cells. They introduced mutations into Num1 that were previously shown to disrupt interactions with mitochondria (Num1-3E). To estimate the effect of the 3E mutations on Num1 clustering, they should directly compare in the same experiment Num1-3E/Mdm36-ox with Num1/Mdm36-ox. Unfortunately, Num1/Mdm36-ox is missing in Fig. 5.*

We apologize for this oversight. In the revised Figure 5A and 5B, we have now included the control Num1-GFP Mdm36^{OX} strain that was imaged in the same experiment (using identical imaging conditions) with the Num1^{3E}-GFP Mdm36^{OX} strain. The results show that, although both Num1^{3E}-GFP and Num1-GFP patches were dramatically enhanced upon Mdm36 overexpression, the levels of the enhanced Num1^{3E}-GFP patches were significantly lower compared to those of Num1-GFP patches in Mdm36^{OX}, indicating that Num1's interaction with mitochondria is indeed required for proper Num1 clustering. We thank the reviewer for the push to strengthen our data.

3. *If mitochondrial interactions are required for Num1 patch formation, and if these interactions are blocked in Num1-3E mutants, it would be expected that the block of mitochondrial interactions also block the formation of large Num1-3E clusters in Mdm36-ox cells, which is obviously not the case. How can this discrepancy be explained? Also, it should be explained how mitochondrial morphology of Num1-3E mutants could be restored upon Mdm36 overexpression if this Num1 variant is unable to interact with mitochondria.*

We would like to clarify that, in addition to mitochondrial interactions, Mdm36 is also required for the formation of large Num1 clusters at the cell cortex. Additionally, although interaction with mitochondria is disrupted in the Num1^{3E} mutants, interaction with Mdm36 is not, as demonstrated previously by Ping et al (see Fig. S3 in JCB 2016, 213:513-524). Thus, conceivably, the appearance of large Num1^{3E} clusters in Mdm36^{OX} cells could be explained by the fact that elevated levels of Mdm36 (upon overexpression) is capable of rescuing Num1 clustering when mitochondrial interactions are impaired by the 3E mutations.
With respect to explaining the mitochondrial morphology in Num1^{3E} mutants, we would like to point out that Ping et al (JCB 2016, 213:513-524) have previously shown that the 3E mutations do not completely abolish the interaction between Num1 and mitochondria. They reported that although the interaction of Num1CC^{3E} (a recombinant CC fragment harboring the 3E mutations) with mitochondrial liposomes was significantly reduced in comparison to that of WT Num1CC, the interaction between Num1CC^{3E} and the liposomes can be restored if the concentration of the mitochondria-specific phospholipid cardiolipin was enhanced in the liposomes (see Fig. 5A in Ping et al JCB 2016). This indicates that the defect introduced by the 3E mutations can be overcome by increasing the binding avidity between Num1 and mitochondria. Thus, we think that the restoration of mitochondrial morphology in Num1^{3E} mutants is due to the assembly of large Num1^{3E} clusters when Mdm36 was overexpressed. In this case, clustering might have increased the avidity of Num1^{3E} for its target membrane, thereby restoring mitochondrial tethering to the cell cortex.

4. Chacko et al. (JCB 2019, 218:3560-71) recently analyzed the role of Mcp5, the Num1 homolog in S. pombe, in tethering of mitochondria during meiosis. They found that the presence of dynein on a Mcp5 cluster precluded the attachment of mitochondria to the same cluster. This work should be discussed and cited.

As requested, we have now discussed and cited the results from Chacko et al. (JCB 2019) in the Discussion section of the revised text (page 18, lines 406-412). Thank you for pointing this out to us.

Minor points

5. It is known that Num1 is asymmetrically distributed between mother and bud in wild type cells, and that it appears in the bud very late in the cell cycle. The authors show that overexpression of Mdm36 leads to the assembly of large Num1 clusters specifically in the mother cell. Do these bright Num1 foci also appear in the bud late in the cell cycle, i.e. in very large buds of Mdm36-ox cells?

We rarely observed bright Num1 foci appearing in small or medium bud of Mdm36^{OX} cells. However, the precise timing for the appearance of bright Num1 foci in large bud is somewhat variable. For instance, in some time-lapse imaging experiments, such as the one shown in Fig. 2B, we observed no bright foci appearing in the bud even at the very late stage of the cell cycle (which we were able to monitor based on spindle morphology in the mRuby2-Tub1 channel) - only dim Num1 patches appeared in the bud during the entire budding process. In other time-lapse imaging experiments, such as the one shown in Video 1, we observed bright Num1 foci appearing in large buds, with similar timing as the Num1 patches observed in WT cells. We also observed bright Num1 foci appearing at the cortex of the newly formed daughter cell, *i.e.* appearing in the next cell cycle after the bud has separated from the mother cell (see Video 1). Thus, the timing for the appearance of bright Num1 foci in the bud might be similar to, or somewhat delayed, relative to the Num1 patches in WT cells.

6. Micrographs should be displayed much larger in Fig. S1B.

We have now enlarged the micrographs in the revised Fig. S1B. Thank you for the suggestion.

7. The authors mention that they observed many Mdm36-ox cells with persistent mitochondrial tether points. It would be nice if they could show representative examples of their videos.

As requested, we have now included a representative video showing persistent mitochondrial tethering to the enhanced Num1 patches in Mdm36^{OX} cells (see new Video 6).

Detailed Point-by-Point Response to Reviewer #3:

We thank the referee for his/her comments and valuable suggestions that helped improve our manuscript.

1. The introduction provides a good, generalized background of the topic. However, the aim of the study is mentioned as, "we set out to characterize the role of Mdm36 in Num1 clustering". This is not correct. Mdm36 is used as a tool to cluster Num1.

We apologize for this error. We have now edited the sentence in the introduction, making it clear that Mdm36 was used as a tool to cluster Num1 in the revised text (page 4, line 77). Thanks for pointing this out to us.

2. The authors could provide more background on how specific experiments were done in order to keep the reader who is not working in this field on board. The GFP counting in Fig. 1 is an example.

As requested, we have now added more details about how specific experiments were performed as we describe the results in the revised text (e.g., lines 106-112 for GFP counting experiment in Fig. 1C, and lines 119-130 for stepwise photobleaching experiment in Fig. S2B and S2C). Thank you for the suggestion.

3. Does num1LL bind dynein when MDM36 is overexpressed? If the authors have the data, it would be good to add to the manuscript.

We have now tagged Dyn1 with 3GFP to assess cortical dynein localization in *num1^{LL}* cells when *MDM36* is overexpressed. The results show that cortical dynein localization was abolished by *num1^{LL}* mutations (0 out of 288 *num1^{LL}* Mdm36^{OX} cells exhibited stationary cortical Dyn1-3GFP foci,

similar to that observed for a *num1*^{LL} strain as reported previously (see Fig. 8A in Tang et al., JCB 2012)), indicating that *num1*^{LL} does not bind dynein when *MDM36* is overexpressed. We thank the reviewer for the push to get these data, which is now described in the revised text (page 10, lines 198-201) and presented as Video 2.

4. The authors describe in results that off-loading of dynein from microtubule plus ends is affected upon MDM36 over-expression. It might be worth to add one or two sentences in the Discussion about this potential mechanism.

As suggested, we have now briefly elaborated on how dynein off-loading might be affected when *MDM36* is overexpressed. We think that one potential explanation is that the number of available offloading sites are reduced when *MDM36* is overexpressed. To ensure a better flow of the text for the reader, we have added a new sentence pertaining to this explanation next to where the data is mentioned in the Results section of the revised text (page 10, lines 191-193). Thank you for the suggestion.

Second decision letter

MS ID#: JOCES/2020/246363

MS TITLE: Overexpression of Mdm36 reveals Num1 foci that mediate dynein-dependent microtubule sliding in budding yeast

AUTHORS: Safia Omer, Katia Brock, John Beckford, and Wei-Lih Lee
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.

Reviewer 1

Advance summary and potential significance to field

In their manuscript entitled “Overexpression of Mdm36 reveals Num1 foci that mediate dynein-dependent microtubule sliding in budding yeast”, Omer et al. demonstrate that a small number of dyneins is sufficient for proper positioning the mitotic spindle. Large Num1 clusters, that are present in Mdm36 overexpressing cells, are seemingly devoid of dynein but instead tether mitochondria. Therefore the authors propose that distinct populations of Num1 serve to tether mitochondria and dynein respectively, with the latter preferring to localize on smaller clusters.

Comments for the author

The authors have addressed all my comments satisfactorily. I recommend publication of this manuscript in JCS. Congratulations on a nice piece of work!

Reviewer 2

Advance summary and potential significance to field

The authors have addressed my previous concerns in an adequate manner.

Comments for the author

I now recommend publication of this interesting manuscript.