Hierarchical integration of mitochondrial and nuclear positioning pathways by the Num1 EF hand

Heidi Anderson, Jason Casler, and Laura Lackner

Corresponding author(s): Laura Lackner, Northwestern

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Reviewer #1 Review (Vaishnavi Ananthanarayanan)

Comments to the Authors (Required):
In this manuscript, Anderson and Lackner set out to understand the role of Num1's EF hand-like motif in anchoring dynein in budding yeast mitosis. In their past work, the authors had demonstrated the requirement for mitochondria-mediated Num1 clustering as a pre-requisite for dynein anchoring, and therefore spindle positioning. In this work, they narrow down this process to Num1's EFLM - by synthetically clustering Num1 (and eliminating the need for mitochondria-mediated clustering), they show that attachment of mitochondria to Num1 alleviated inhibition of dynein binding in a manner dependent on the ELFM. While this study has been carefully performed, the primary concern this reviewer has with the manuscript is that it is a rehash of an earlier paper by the authors (Schmit et al., Cell Cycle 2018), now with just the inclusion of the EFLM mutants. There is also no mechanistic insight into how the EFLM prevents association of dynein with Num1 without mitochondrial interaction. My specific comments appear below:

Major comments:
1. Given that Num1-CC-yEGFP or Num1-delPH-yEGFP are produced from the endogenous Num1 locus, the total intensity of GFP in the PAN or PAN-CC constructs must be similar to Num1-GFP or Num1 CC-GFP, regardless of the number of potential eisosome sites on the PM. Can the authors quantify this and show this to be the case?

2. The authors posit that "Importantly, similar to PAN clusters but distinct from WT Num1 clusters, the majority of PAN-CC clusters are not associated with mitochondria" - isn't this expected given the number of clusters in PAN and PAN-CC? The limiting factor here would be the amount of mitochondria. It would be better to measure number of clusters per length of mitochondrion.

3. In Fig. 2D, why are the representative images shown that of dynein, mito and Num1/PANdelEF in the mother cells? The entire cell should be shown, and importantly the bud.

4. The total number of cortical dynein spots in all mutants needs to be measured. Is there a difference? Following this, the % of cortical dynein not assoc with mito can be measured.

5. In for data in Fig 3. and 4, simultaneous visualisation of dynein and Num1 clusters (synthetic and WT + mutants) needs to performed.

6. It is unclear to this reviewer why "The level of Num1-CC in PAN-CC cells was ~5 times greater". Soon after, the authors propose that "the increase in cortical dynein anchored at clusters without mitochondria in PAN-CC, PANΔEF, and PAND315A cells cannot be attributed to changes in Num1 protein levels". It is confusing as to how this conclusion was made.

7. While it is apparent that the EFLM responds to Ca2+ in vitro, it has not been shown if this function is required in vivo and what its role might be.

8. How was the decision to perform a parametric test for significance made? What was the test for normality that was performed?

Minor comments:
1. The manuscript was hard to read. Portions of Figs. 1, 2 and 3 are referred to much later in the manuscript and therefore require going back and forth with the figures. I would recommend splitting the figures in such a way as to avoid this.

2. There is too much of the results of this study in the Introduction. I would recommend removing this.

3. Some data have been represented in bar plots and others in scatter plots with means. For uniformity and transparency, all data should be represented in scatter plots.

4. The schematic in Fig. 6 does not convey any information that was not found in another paper by the authors (Kraft et al., 2017), except perhaps the ER attachment, which is not central to this work. I would therefore recommend removing it.

Reviewer #2 Review (Koji Okamoto)

Comments to the Authors (Required):
In this manuscript, Anderson and Lackner report a previously unappreciated role for the EFLM (EF hand-like motif) of Num1 in regulation of dynein function upon mitochondrial inheritance in budding yeast. Previous studies reveal that mitochondrial positioning at the right place and timing requires Num1, a multidomain protein of 313 kDa, to form clusters at the cell cortex. This cortical Num1 cluster formation is mediated by its direct interaction with mitochondria. In addition, the cortical Num1 clusters (but its monomer does not) anchor the microtubule-associated motor protein dynein to promote spindle migration for nuclear positioning. Accordingly, it has been hypothesized that mitochondria serve as platforms for Num1 to form clusters capable of anchoring dynein for spindle migration, thereby biasing nuclear positioning to occur subsequent to mitochondrial positioning. However, there are studies that provide the data against this hypothesis, and the molecular mechanisms underlying Num1-driven nuclear inheritance coupled to prior mitochondrial inheritance remain to be elucidated. Using a synthetic mitochondria-independent cortical Num1 clustering system and genetically manipulated strain with conditional disruption of bud-directed mitochondrial positioning, the authors found that mitochondria-associated Num1 clusters predominantly anchor dynein in a manner dependent on the EFLM, and that loss of the EFLM leads to a rescue in dynein-mediated spindle migration even in the absence of mitochondrial inheritance. Furthermore, isothermal titration calorimetry assays for recombinant Num1(97-324) variants containing the EFLM indicated that Num1 is a bona fide calcium-binding protein. Collectively, the authors propose that the EFLM binds calcium ions to bias dynein anchoring towards mitochondria-associated cortical Num1 clusters, acting as a surveillance system to ensure mitochondrial inheritance prior to nuclear inheritance.

The data in this study are of high quality and potentially interesting, which could provide new insights into the molecular understanding of organelle inheritance. However, it still remains unclear how calcium binding leads to enhanced anchoring of dynein to mitochondria-associated Num1 clusters. In particular, does calcium-bound Num1 have an increased affinity for Mdm36, a component of the mitochondria-ER-cortex-anchor that promote mitochondria-associated Num1 clustering? Does calcium efflux from the ER affect Num1 for dynein anchoring? Without addressing these issues, the current manuscript represents a more limited advance than the previously published papers. Finally, this study would significantly be strengthened if the authors also clarify the following points.

Additional points:

1. How can the EFLM mutants form clusters to anchor dynein for spindle migration in the absence of mitochondrial inheritance (Figure 4G)? The authors should add microscopic data on cells expressing mitochondrial marker and GFP-tagged Num1 wild-type or the EFLM mutants with or without mitochondrial inheritance (as shown in Figure 3A), and explain about mitochondria-independent Num1 EFLM mutant clustering in the bud.

2. In Figure 6, the authors should add a description of each panel of the diagrams to make it easier for the readers to understand the model.

3. The authors should add line numbers in each page, so that referees can easily point out sentences where they have something to ask.

Reviewer #3 Review

Comments to the Authors (Required):
This manuscript aimed to dissect the function of Num1. The authors successfully charactering an EF hand-like motif (EFLM) that can bind calcium. The results demonstrated that EFLM is critical for dynein anchoring rather than mitochondria association based on mitochondrial-associated clusters and spindle formation. Following the characterization, the authors argued that the bias of dynein anchoring on mitochondria associated Num1 clusters indicated an integration of mitochondrial and nuclear

positioning pathway exists. This hierarchical positioning of organelles may play a role in organelle inheritance during cell cycle. The experimental design is elegant, and the data is clearly presented. The manuscript fits into the journal aims and scope. General readers will be interested in the approaches the authors offered.

Major concern:
1. One of the concerns about the manuscript is that the authors did not address the possibility of "gain-of-function" for expressing truncated/mutated EFLM in the experiments. One example is the Figure 3. It is nice to see the addition of auxin had no effect on spindle orientation in PANΔEF and PAND315A cells. However, different interpretations of this results exist. So is Figure 4. It is the reviewer's opinion that the authors should address this issue in the interpretations of results throughout the manuscript.
2. The authors examined the effects of EFLM truncated/mutant on the associate of Num1 and ER. It is rather difficult for the reviewer to distinguish the difference between WT and Δlnp1 strains based on the current quality of Figure 5A image qualify. The help from authors to guide through the image will aid general readers.
3. It is the reviewer's opinion that authors should examine the effects of calcium signaling on Num1 function. This may lead to more understanding of the role of EFLM in organelle inheritance and others.

Minor concerns
1. Page 6, line 1. Typo: "...when the EFLM is disrupted,".
2. Page 10, last line. Typo: "...synthetically clustered EFLM mutants (Fig. 2D and E).".
3. Page 11, line 7. Typo: "... clustered EFLM mutants.".
4. Page 11, line 10. Grammar error. "...the function of the EFLM was still unclear." The sentence could be more directly such as: The function of the EFLM was still unclear based on the available results.

RE: Manuscript #E21-12-0610-T
TITLE: "Hierarchical integration of mitochondrial and nuclear positioning pathways by the Num1 EF hand"

Dear Prof. Lackner:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Martin Ott
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Lackner:

Congratulations on the acceptance of your manuscript.

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

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Sincerely,

Eric Baker
Journal Production Manager
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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 are still actively working to gain a better understanding of the role cellular calcium plays in the regulation of Num1. This role is not straightforward to address due to complications from indirect effects many Ca^{2+}-related mutants have. Therefore, this work will take time to complete and will be the focus of a future manuscript.

We strongly feel that our revised manuscript provides a substantial advance in our understanding of mitochondria-dependent dynein anchoring and the role of the Num1 EFLM, the function of which was previously unknown, in this process. Specifically, our study provides the following novel insights:
  - We demonstrate, for the first time, that the Num1 EFLM is a bona fide Ca^{2+}-binding EF hand.
  - We demonstrate that Ca^{2+}-binding by the EF is required to bias dynein anchoring on mitochondria-assembled and -associated Num1 clusters.
  - We find that mitochondria are not required for dynein function post-anchoring, which addresses an outstanding question about the function of mitochondria-dependent dynein anchoring.
  - Our findings suggest a model in which the coupling of dynein anchoring and, consequently, dynein-mediated nuclear inheritance to the presence of mitochondria in the bud provides a spatiotemporal mechanism of organelle inheritance that favors mitochondrial inheritance prior to that of the nucleus and may function as a mitochondrial inheritance surveillance system.

Point-by-point response:
Reviewer #1
General comments: *In this manuscript, Anderson and Lackner set out to understand the role of Num1's EF hand-like motif in anchoring dynein in budding yeast mitosis. In their past work, the authors had demonstrated the requirement for mitochondria-mediated Num1 clustering as a pre-requisite for dynein anchoring, and therefore spindle positioning. In this work, they narrow down this process to Num1's EFLM - by synthetically clustering Num1 (and eliminating the need for mitochondria-mediated clustering), they show that attachment of mitochondria to Num1 alleviated inhibition of dynein binding in a manner dependent on the ELFM. While this study has been carefully performed, the primary concern this reviewer has with the manuscript is that it is a rehash of an earlier paper by the authors (Schmit et al., Cell Cycle 2018), now with just the inclusion of the EFLM mutants. There is also no mechanistic insight into how the EFLM prevents association of dynein with Num1 without mitochondrial interaction. My specific comments appear below:*

Major comments:
1. Given that Num1-CC-yEGFP or Num1-delPH-yEGFP are produced from the endogenous Num1 locus, the total intensity of GFP in the PAN or PAN-CC constructs must be similar to Num1-

In the original manuscript, we quantified protein levels via western blot (see Fig. S1) and found that the levels of Num1ΔPH and PAN are less than 2-fold higher than WT Num1 and the levels of Num1-CC and PAN-CC ~5 fold higher than the WT. Based on the reviewer's suggestion, we also quantified protein levels on a single cell level by measuring the total GFP intensity per cell and find that the trends are similar (see reviewer Fig 1). Given that the quantification of protein levels from western blots was already included in the manuscript and the whole cell GFP intensity measurements do not add to or alter the findings of our study in any way, we have not included GFP intensity measurement data in the manuscript. However, we would be happy to include the data should it be deemed necessary.
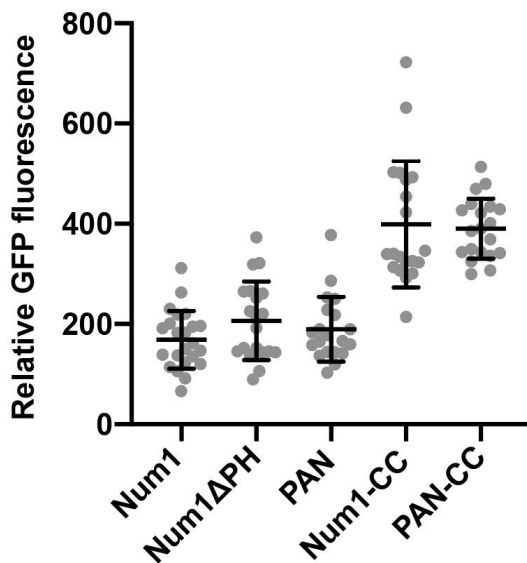

Figure 1. Quantification of whole cell GFP intensity for Num1, Num1ΔPH, PAN, Num1-CC, and PAN-CC. A ROI was manually drawn around a single cell to obtain the mean gray value and area. Background fluorescence was obtained from an average of three ROIs per image in regions that did not contain cells. The fluorescence was normalized by subtracting the background fluorescence from the mean gray value and then divided by the total area of the cell. n ≥ 20 cells.

2. The authors posit that "Importantly, similar to PAN clusters but distinct from WT Num1 clusters, the majority of PAN-CC clusters are not associated with mitochondria" - isn't this expected given the number of clusters in PAN and PAN-CC? The limiting factor here would be the amount of mitochondria. It would be better to measure number of clusters per length of mitochondrion.

The reviewer is correct — it is expected that the majority of PAN and PAN-CC clusters are not associated with mitochondria. That is the reason we are using the artificial clustering system. It

serves as a tool to assemble Num1 clusters in the absence of mitochondria and create a pool of clusters that are not mitochondria associated.

We have modified the text in two areas to help make that point more clear (modifications are shown in blue):

Truncated versions of Num1 are then expressed as yEGFP fusions from the endogenous *NUM1* locus in the cells expressing Pil1-αGFP, resulting in recruitment of Num1 to eisosomes and its clustering on the PM (Fig. 1A and C; Schmit *et al.*, 2018). The synthetic clustering system bypasses the need for mitochondrial association in cluster formation, allowing us to create populations of Num1 that are and are not associated with mitochondria. Thus, we can use this system as a tool to uncouple Num1 cluster formation at the PM from mitochondria association. Cells expressing the components of the synthetic clustering system are referred to as *PAN* (for Pil1-associated Num1) cells and the synthetic protein clusters are referred to as PAN clusters.

To test this, we expressed Num1(aa1-303)-yEGFP (Num1-CC) in cells expressing Pil1-αGFP (Num1-CC-yEGFP Pil1-αGFP, referred to as PAN-CC; Fig. 1B). Importantly, we found that while PAN-CC clusters can tether mitochondria to the PM, most PAN-CC clusters are not associated with mitochondria (Fig. 1C-E). This is similar to PAN clusters, in which Num1ΔPH-yEGFP is synthetically clustered, but distinct from WT Num1 clusters where the majority of clusters are associated with mitochondria (Fig. 1C-E). There is also an overall increase in the number of mitochondria-associated clusters per cell for PAN and PAN-CC compared to WT Num1. This increase is expected due to the increased number of total PAN and PAN-CC clusters per cell, a result of targeting the Num1 truncations to eisosomes, which are highly abundant on the PM (Fig. 1D and E; Walther *et al.*, 2006; Schmit *et al.*, 2018). ~~Importantly, similar to PAN clusters but distinct from WT Num1 clusters, the majority of PAN-CC clusters are not associated with mitochondria (Fig. 1D-F).~~

3. In Fig. 2D, why are the representative images shown that of dynein, mito and Num1/PANdelEF in the mother cells? The entire cell should be shown, and importantly the bud.

We have expanded the region shown in the images to now include the whole cell (mother and bud). We have also included an example of dynein anchored in the bud for PANΔEF cells. The vast majority of cortically anchored dynein foci are found in the mother cell and are rarely observed in buds (Lee et al. 2005). Therefore, showing dynein anchoring events in mother cells is an accurate representation of what we observe when we image cells.

4. The total number of cortical dynein spots in all mutants needs to be measured. Is there a difference? Following this, the % of cortical dynein not assoc with mito can be measured.

The total number of cortical dynein spots was measured and used to calculate the percent of cortical dynein not associated with mitochondria. In the revised manuscript, we have now added the data regarding the percent of cells in which a cortical dynein focus is observed to the results section as follows (added text is shown in blue):

We observed a cortical dynein focus in 42.1% of *PAN-CC* cells (n = 152 cells), which is a dramatic increase in comparison to WT and *PAN* cells in which cortically anchored dynein was observed in 2.5% and 16.5% of cells, respectively (n = 320 and 284 cells, respectively).

We observed cortical dynein anchoring events in 15.8% and 20.3% of *PANΔEF* and *PAN^{D315A}* cells respectively (n = 558 and 295, respectively), similar to *PAN* cells (16.5%, n = 284).

The fact that we observe cortical dynein foci in a higher percentage of cells expressing PAN-CC than in PAN, PANΔEF, and PAN^{D315A}, could be a result of dysregulation of dynein anchoring in these cells by the truncated version of Num1 and/or the fact that the levels of Num1-CC in *PAN-CC* cells are higher than the Num1 levels in *PAN*, *PANΔEF,* and *PAN^{D315A}* cells. However, the important comparisons for our studies are the dynein anchoring events in *PAN*, *PANΔEF,* and *PAN^{D315A}* cells, for which the percent of cells in which cortical dynein foci are observed are similar but the percent of cortical dynein not associated with mitochondria differ.

5. In for data in Fig 3. and 4, simultaneous visualisation of dynein and Num1 clusters (synthetic and WT + mutants) needs to be performed.

In the original manuscript, the focus of Figs. 3 and 4 (Figs. 4 and 6 in the revised version) was assessing dynein function in the absence of mitochondrial inheritance in various artificially clustered (now Fig. 4) and full length (now Fig. 6) Num1 mutants. Dynein function was assessed using a spindle orientation assay and, importantly, a spindle oscillation assay, which is a more direct and sensitive measurement of dynein function in the bud than direct visualization of dynein.

For the revised manuscript, we simultaneously imaged dynein, Num1, and mitochondria in both WT and *Num1^{D315A} mito^{AID}* cells treated with auxin to inhibit mitochondrial inheritance. While the vast majority of cortically anchored dynein foci are found in the mother cell and are rarely observed in buds (Lee et al. 2005), we were able to capture examples of cortical dynein anchoring in the bud. We have added those data as Fig. 6C and discuss those data in the last paragraph of the results section. The fact that we were able to capture more examples of cortically anchored dynein in the buds of cells expressing Num1^{D315A} than in the buds of WT cells is consistent with our findings that we observe properly orientated spindles as well as spindle oscillation in a higher percentage of cells expressing Num1^{D315A} in comparison to WT Num1 in the absence of mitochondria.

6. It is unclear to this reviewer why "The level of Num1-CC in PAN-CC cells was ~5 times greater". Soon after, the authors propose that "the increase in cortical dynein anchored at clusters without mitochondria in PAN-CC, PANΔEF, and PAND315A cells cannot be attributed to changes in Num1 protein levels". It is confusing as to how this conclusion was made.

We thank the reviewer for pointing out their confusion and agree that the wording we used was confusing. We have modified the text as follows for clarification (modifications are shown in blue): While Num1 protein levels for Num1-CC in *PAN-CC* cells was ~5 times greater than the Num1

protein levels in PAN cells, the Num1 protein levels in *PANΔEF* and *PAN*[D315A] cells were comparable to the Num1 protein levels in *PAN* cells (Fig. S1A and B). Thus, the increase in cortical dynein anchored at clusters without mitochondria in *PAN-CC*, *PANΔEF,* and *PAN*[D315A] cells cannot be attributed to changes in Num1 protein levels alone. Together, the data indicate that, by disrupting the EFLM in our synthetic clustering system, the bias of anchoring dynein on mitochondrial-associated clusters is reduced. These results support the hypothesis that the EFLM is important for the regulation of mitochondria-biased dynein anchoring.

7. While it is apparent that the EFLM responds to Ca2+ in vitro, it has not been shown if this function is required in vivo and what its role might be.

We have been able to show in vitro that the EFLM binds Ca^{2+} and that the D315A EFLM mutant is not able to bind Ca^{2+}. When we express the calcium binding mutant, $Num1^{D315A}$, in cells, we find that Num1-mediated dynein anchoring is no longer biased by the presence of mitochondria, suggesting the bias is dependent on the ability of the EFLM of Num1 to bind Ca^{2+}. We are working to gain a better understanding of the role cellular calcium plays in the regulation of Num1, which will be the focus of a future manuscript.

8. How was the decision to perform a parametric test for significance made? What was the test for normality that was performed?

We thank the reviewer for pointing out that we did not include enough information regarding the statistical analyses performed and apologize for this oversight. We have now updated the methods as follows (modifications are shown in blue):
Statistical analyses were performed in GraphPad using a two-tailed unpaired t-test with a 95% confidence interval (the Shapiro-Wilk test was used to test for normality), with exception to Figures 1D and 1E, for which the Mann-Whitney test with a 95% confidence interval was used. Designations of significance are indicated in the figure legends.

Minor comments:
1. The manuscript was hard to read. Portions of Figs. 1, 2 and 3 are referred to much later in the manuscript and therefore require going back and forth with the figures. I would recommend splitting the figures in such a way as to avoid this.

We appreciate the reviewer's comment and have tried to organize the paper in many ways. We decided on the format we chose to keep similar experiments together. Fig. 1 and Fig. 2 include data for the artificial Num1 clusters with Fig. 1 describing their association with mitochondria and Fig. 2 focusing on their ability to anchor dynein and support dynein function. If we split the PAN-CC data and PANΔEF/PAN[D315A] data into separate figures, we would need to show the same controls for specific experiments in multiple figures and it would be difficult to make comparisons between the mutants for the specific functions being assayed in each experiment. We did decide to move the biochemistry experiments to Fig. 3 to demonstrate earlier in the paper that the EFLM

does indeed bind Ca^{2+} and that the D315A mutant disrupts Ca^{2+}-binding. We think that change helps the flow of the paper and better sets the stage for the data shown in Figs. 4-6.

Fig. 3, which is now Fig. 4 in the revised version, is not called out in the manuscript after its initial discussion in the results section. For Figs. 1 and 2, there are 3 and 1 callouts, respectively, later in the manuscript to remind the reader of the data presented and discussed earlier in the manuscript.

2. There is too much of the results of this study in the Introduction. I would recommend removing this.

In the revised manuscript, we removed some of the discussion of results from the last paragraph of the introduction and as a result have shortened that paragraph.

3. Some data have been represented in bar plots and others in scatter plots with means. For uniformity and transparency, all data should be represented in scatter plots.

For data in which at least 3 independent experiments were conducted, the data point for each independent experiment is shown. However, for the graphs that depict data for the % cortical dynein not associated with mitochondria and the spindle oscillation assays (Figs. 2E, 5E and F, and 6B), the data are collected over the course of multiple days and imaging sessions until an n of at least 100 cortical dynein foci or 270 spindles is reached. These experiments require long-term time lapse imaging and it is not possible to achieve large n's in one imaging session. Therefore, the data from multiple sessions are aggregated into one final number. To better clarify the total numbers of cortical dynein foci or spindles that went into each data set, we have added n's above each bar on the graphs shown in Figs. 2E, 5E and F, and 6B.

4. The schematic in Fig. 6 does not convey any information that was not found in another paper by the authors (Kraft et al., 2017), except perhaps the ER attachment, which is not central to this work. I would therefore recommend removing it.

We have added more detail to the model figure, which is now Fig. 7 in the revised manuscript, to better highlight the role for a functional EFLM (i.e. an EFLM that can bind Ca^{2+}) in the process of mitochondria-dependent dynein anchoring and timing of organelle inheritance.

Reviewer #2

General comments: *In this manuscript, Anderson and Lackner report a previously unappreciated role for the EFLM (EF hand-like motif) of Num1 in regulation of dynein function upon mitochondrial inheritance in budding yeast. Previous studies reveal that mitochondrial positioning at the right place and timing requires Num1, a multidomain protein of 313 kDa, to form clusters at the cell cortex. This cortical Num1 cluster formation is mediated by its direct interaction with mitochondria. In addition, the cortical Num1 clusters (but its monomer does not) anchor the microtubule-associated motor protein dynein to promote spindle migration for nuclear positioning. Accordingly,*

it has been hypothesized that mitochondria serve as platforms for Num1 to form clusters capable of anchoring dynein for spindle migration, thereby biasing nuclear positioning to occur subsequent to mitochondrial positioning. However, there are studies that provide the data against this hypothesis, and the molecular mechanisms underlying Num1-driven nuclear inheritance coupled to prior mitochondrial inheritance remain to be elucidated. Using a synthetic mitochondria-independent cortical Num1 clustering system and genetically manipulated strain with conditional disruption of bud-directed mitochondrial positioning, the authors found that mitochondria-associated Num1 clusters predominantly anchor dynein in a manner dependent on the EFLM, and that loss of the EFLM leads to a rescue in dynein-mediated spindle migration even in the absence of mitochondrial inheritance. Furthermore, isothermal titration calorimetry assays for recombinant Num1(97-324) variants containing the EFLM indicated that Num1 is a bona fide calcium-binding protein. Collectively, the authors propose that the EFLM binds calcium ions to bias dynein anchoring towards mitochondria-associated cortical Num1 clusters, acting as a surveillance system to ensure mitochondrial inheritance prior to nuclear inheritance.

The data in this study are of high quality and potentially interesting, which could provide new insights into the molecular understanding of organelle inheritance. However, it still remains unclear how calcium binding leads to enhanced anchoring of dynein to mitochondria-associated Num1 clusters. In particular, does calcium-bound Num1 have an increased affinity for Mdm36, a component of the mitochondria-ER-cortex-anchor that promote mitochondria-associated Num1 clustering? Does calcium efflux from the ER affect Num1 for dynein anchoring? Without addressing these issues, the current manuscript represents a more limited advance than the previously published papers. Finally, this study would significantly be strengthened if the authors also clarify the following points.

We thank the reviewer for the overall positive comments about the manuscript and data. We are actively working to address the exciting questions the reviewer outlines to gain a better understanding of the role cellular calcium plays in the regulation of Num1. However, as mentioned above, these questions are not straightforward to address due to complications from indirect effects many Ca^{2+}-related mutants have. Therefore, this work will take time and be the focus of a future manuscript.

We do feel that our current study provides a substantial advance in our understanding of the EFLM of Num1, the function of which was previously unknown, and its role in mitochondria-dependent dynein anchoring, and have highlighted the novel insights our study provides in the introductory response above.

Additional points:

1. How can the EFLM mutants form clusters to anchor dynein for spindle migration in the absence of mitochondrial inheritance (Figure 4G)? The authors should add microscopic data on cells expressing mitochondrial marker and GFP-tagged Num1 wild-type or the EFLM mutants with or without mitochondrial inheritance (as shown in Figure 3A), and explain about mitochondria-independent Num1 EFLM mutant clustering in the bud.

In the revised manuscript, we simultaneously imaged dynein, Num1, and mitochondria in both WT and *Num1^D315A^ mito^AID^* cells treated with auxin to inhibit mitochondrial inheritance. While the vast majority of cortically anchored dynein foci are found in the mother cell and are rarely observed in buds (Lee et al. 2005), we were able to capture examples of cortical dynein anchoring in the bud. We have added those data as Fig. 6C and discuss those data in the last paragraph of the results section. The Num1 assemblies present at the sites of cortical dynein anchoring were smaller and more diffuse than mitochondria-assembled Num1 clusters, consistent with previous descriptions of dynein anchoring by Num1 in the absence of mitochondria (Omer et al. 2018; Omer et al. 2020). The fact that we were able to capture more examples of cortically anchored dynein in the buds of cells expressing Num1^D315A^ than in the buds of WT cells is consistent with our findings that we observe properly orientated spindles as well as spindle oscillation in a higher percentage of cells expressing Num1^D315A^ in comparison to WT Num1 in the absence of mitochondria.

2. In Figure 6, the authors should add a description of each panel of the diagrams to make it easier for the readers to understand the model.

We have added more detail to the model figure, which is now Fig. 7 in the revised manuscript, to better highlight the role for a functional EFLM (i.e. an EFLM that can bind Ca^{2+}) in the process of mitochondria-dependent dynein anchoring and timing of organelle inheritance.

3. The authors should add line numbers in each page, so that referees can easily point out sentences where they have something to ask.

We apologize for this oversight. Line numbers have been added to the revised manuscript.

Reviewer #3
General comments: *This manuscript aimed to dissect the function of Num1. The authors successfully charactering an EF hand-like motif (EFLM) that can bind calcium. The results demonstrated that EFLM is critical for dynein anchoring rather than mitochondria association based on mitochondrial-associated clusters and spindle formation. Following the characterization, the authors argued that the bias of dynein anchoring on mitochondria associated Num1 clusters indicated an integration of mitochondrial and nuclear positioning pathway exists. This hierarchical positioning of organelles may play a role in organelle inheritance during cell cycle. The experimental design is elegant, and the data is clearly presented. The manuscript fits into the journal aims and scope. General readers will be interested in the approaches the authors offered.*

Major concerns:
1. One of the concerns about the manuscript is that the authors did not address the possibility of "gain-of-function" for expressing truncated/mutated EFLM in the experiments. One example is the Figure 3. It is nice to see the addition of auxin had no effect on spindle orientation in PANΔEF and PAND315A cells. However, different interpretations of this results exist. So is Figure 4. It is

In the discussion, we now discuss the possibility that activity of the Num1 EFLM represents a gain-of-function phenotype. The following modifications (shown in blue) were made to the text: Our findings suggest a direct role for the Num1 EFLM in mitochondria-dependent dynein anchoring. Specifically we find that, in the presence of a functional EFLM, Num1-dependent anchoring of dynein is heavily weighted towards Num1 clusters that are associated with mitochondria and this bias is reduced when the EFLM is disrupted. Consistently, when the EFLM is disrupted, we do not observe defects in dynein-dependent spindle orientation and oscillation in the absence of mitochondrial inheritance. These results suggest that the EFLM negatively impacts the association of dynein with Num1 prior to the association of Num1 with mitochondria and that the Num1-mitochondria association relieves the negative effect of the EFLM. At this point, we cannot rule out an alternative explanation in which disrupting the EFLM results in a gain of function phenotype that promotes dynein anchoring by Num1 proteins that are not associated with mitochondria. However, in either scenario, the presence of a functional EFLM serves to couple dynein-mediated nuclear inheritance to mitochondrial inheritance.

2. The authors examined the effects of EFLM truncated/mutant on the associate of Num1 and ER. It is rather difficult for the reviewer to distinguish the difference between WT and Δlnp1 strains based on the current quality of Figure 5A image qualify. The help from authors to guide through the image will aid general readers.

We thank the reviewer for pointing out their confusion over the ER images and agree that the wording we used was confusing. We have expanded the description and provided more detail for the images for what was Fig. 5A but is now Fig. 5G. We modified the text as follows for clarification (modifications are shown in blue):

We also examined the relationship between full length Num1 EFLM mutant clusters and the ER, which is a component of MECA. Interestingly, the ER can locally concentrate calcium levels (Cunningham, 2011). Thus, it is possible that calcium binding by the EFLM could be regulated by association with the ER. Additionally, the EFLM region of Num1 contains a putative FFAT motif, composed of amino acids 306-330 (Fig. 1B), which has been suggested to mediate the interaction between Num1 and the integral ER protein Scs2 (Chao *et al.*, 2014). However, our previous work indicates that versions of Num1 that lack the EFLM are still able to associate with the ER (Lackner *et al.*, 2013), and there is debate about whether amino acids 306-330 compose a bona fide FFAT motif (Chao *et al.*, 2014; Murphy and Levine, 2016). To determine whether *NUM1ΔEF and NUM1^{D315A}* cells have a defect in the association of Num1 with the ER resulting from disruption of the putative FFAT motif, we examined the localization of Num1ΔEF and Num1^{D315A} relative to the ER (Fig. 5G). Similar to Num1 clusters, Num1ΔEF and Num1^{D315A} clusters localized to regions of the cell cortex that are occupied by cortical ER (Fig. 5G left). Since the cortical ER covers a significant portion of the cell cortex, we also examined Num1-ER localization in *Δlnp1* cells that disrupts cortical ER localization and produces large regions of the cortex devoid of cortical ER (Chen *et al.*, 2012). In this background, Num1 association with the ER should be more apparent. Indeed, the ER association for WT Num1, Num1ΔEF and Num1^{D315A} clusters was highly evident

in the Δ*lnp1* background (Fig. 5G right). Furthermore, when the Num1-ER association is disrupted through deletion of Scs2, Num1 clustering is affected (Chao *et al.*, 2014; Omer *et al.*, 2018). However, we observe no obvious clustering defects for Num1ΔEF and Num1^{D315A} (Fig. 5A), further suggesting that the EFLM likely does not interact with the ER through Scs2. These results indicate that the deletion of the EFLM or introduction of the D315A mutation does not interfere with the association of Num1 clusters with the ER.

3. It is the reviewer's opinion that authors should examine the effects of calcium signaling on Num1 function. This may lead to more understanding of the role of EFLM in organelle inheritance and others.

We agree with the reviewer that understanding the mechanism by which calcium and calcium signaling affect Num1 function would lead to a better understanding of the role of the EFLM in coordinated organelle inheritance. As mentioned above, we are still actively working to gain a better understanding of the role cellular calcium plays in the regulation of Num1. This role has not been straightforward to address due to complications from indirect effects many Ca^{2+}-related mutants have. Therefore, this work will take time to complete and will be the focus of a future manuscript.

Minor concerns:
1. Page 6, line 1. Typo: "...when the EFLM is disrupted,".
2. Page 10, last line. Typo: "...synthetically clustered EFLM mutants (Fig. 2D and E).".
3. Page 11, line 7. Typo: "... clustered EFLM mutants.".
4. Page 11, line 10. Grammar error. "...the function of the EFLM was still unclear." The sentence could be more directly such as: The function of the EFLM was still unclear based on the available results.

We thank the reviewer for catching and pointing out these typos and errors. All have been corrected.

RE: Manuscript #E21-12-0610-T
TITLE: "Hierarchical integration of mitochondrial and nuclear positioning pathways by the Num1 EF hand"

Dear Prof. Lackner:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Martin Ott
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Lackner:

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