The microtubule-associated protein She1 coordinates directional spindle positioning by spatially restricting dynein activity

Kari H. Ecklund, Megan E. Bailey, Kelly A. Kossen, Carsten K. Dietvorst, Charles L. Asbury and Steven M. Markus

DOI: 10.1242/jcs.258510

Editor: David Glover

Review timeline

Original submission:	8 February 2021
Editorial decision:	9 March 2021
First revision received:	15 September 2021
Editorial decision:	25 October 2021
Second revision received:	25 October 2021
Accepted:	27 October 2021

Original submission

First decision letter

MS ID#: JOCES/2021/258510

MS TITLE: The MAP She1 coordinates directional spindle positioning by spatially restricting dynein activity

AUTHORS: Kari H. Ecklund, Megan E. Bailey, Carsten K. Dietvorst, Charles L. Asbury, and Steven M. Markus
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.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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.

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

This manuscript addresses the mechanism of She1's action in yeast spindle positioning. She 1 is a budding yeast-specific MAP whose deletion affects dynein-mediated spindle positioning. She1's role was initially implicated in controlling the timing of the dynein-dynactin interaction in vivo and then in enhancing dynein-microtubule interaction and inhibiting dynein motility in vitro. In this study, the authors found that she1 induces a persistent force generating state for dynein in vitro, consistent with a stronger dynein-microtubule interaction. However, the model that She1 enhances dynein force generation to help spindle movement toward the bud does not fit well with their current in vivo data (especially since the requirement for She1 was only mildly affected by the loss of Bni1 (formin) that produces a wider bud neck). They then performed a series of in vivo experiments to examine the possible mechanism of She1 in directional spindle movement toward the bud. Their new data show that She1 preferentially localizes along astral microtubules in the mother cell compartment and inhibits the initiation of dynein-mediated spindle movement in the mother cell compartment. They also show that this action of She1 depends on the microtubule-binding state of dynein via its microtubule-binding domain. In addition, they also show that nuclear She1 is not involved in this function.
Moreover, their data also suggest a role of She1 in the spindle-positioning checkpoint.

Overall, I found this study very well performed, and its mechanistic insights are clear. It emphasizes the importance of performing a mechanistic study in the in vivo context as their previous work on She1's effect on purified dynein alone did not lead to a model that fits with their in vivo data. The authors examined the existing model critically and carefully with a series of well-designed experiments, and their work led to a new model of She1 function. Although She1 is yeast-specific, a general principle of how a MAP may affect motor function is applicable to other organisms. Thus, I believe that this paper makes a significant contribution to our understanding of cell biology and is of broad interest to the cell biology community.
The paper is written clearly and contains very interesting discussions. I fully support its publication but would provide the following Comments for the authors to consider.

Comments for the author

1. Most of the experiments were done in the kar9Δ background, and do you see the oscillatory spindle movements across the neck as first described by Yeh et al., 2000 MBoC? If so, it would be better to mention that. I think that in the Moore et al., 2009 PNAS study, the authors also used the kar9Δ background to detect a function of p150 GAP-Gly. On page 15, you wrote that "our analysis of spindle movements in nip100ΔCAP-gly mutant cells revealed no significant impact on dynein function (Fig. 4A – H). These data contrast with prior observations38." Did you use the kar9Δ background for the experiments described in Fig. 4? It would be better to clarify this point and modify the conclusion if necessary.

2. Page 25, "Thus, the She1-dynein MTBD contacts may directly promote the autoinhibited conformation, which is mediated in part by contact points in the coiled-coil stalk within the heavy chain46,54," This is an interesting point although speculative. I would suggest adding a paper by "Niekamp et al., 2019 EMBO" here to say that changes in the stalk can indeed be transmitted to the motor ring. Potentially, you could check if the dynein-LIS1 interaction is affected by She1 and if introducing the phi mutation that keeps dynein open would make a difference. While these experiments are not essential for the main conclusion of the current paper, a more detailed discussion on this point including the auto-inhibited phi vs. open conformation of dynein and LIS1's role in promoting the open state as revealed recently in yeast, Aspergillus, in vitro, should help the reader understand this point more easily. In addition could it also be possible that dynein-dynactin simply fall apart during offloading if dynein gets stuck on the microtubule for too long?

3. Here I would make several minor suggestions on the text:
(1) Abstract, “dynein microtubule-binding domain, and to astral microtubules”, the comma can be removed. It would also be better to say “and its preferential binding to astral…”.
(2) Page 4, “orthologous to NuMA in humans”. You may consider adding “Greenberg et al., 2018, Biophys Rev” as a reference.
(3) Page 4, “a greater number of dynein foci are apparent in mother cells,” Please add a reference.
(4) Page 6, “mispositioned spindles trigger a spindle positioning checkpoint (SPC),” you may consider adding “Yeh et al., 1995 JCB” as an original reference.
(5) Page 8 “KAR9, a key member of an actomyosin-mediated spindle orientation pathway37.” You may consider adding “Miller and Rose 1998 JCB” as an original reference.
(6) Page 13 “spindle microtubule stability43,” should be changed to “spindle stability43”

Reviewer 2

Advance summary and potential significance to field

How Microtubule Associated Proteins (MAPs) regulate motors that move along microtubules is an important question, and one that is currently attracting attention in the microtubule field. There are clear examples of MAPs inhibiting motor activity in vitro and in some cases this appears to be specific for certain MAPs inhibiting certain motors. For the most part, the mechanistic details remain unclear and how this regulation impacts motor function in cells is understudied.

This study by Ecklund and colleagues addresses these knowledge gaps by investigating the She1 protein, a MAP that regulates dynein activity in budding yeast. She1 is a yeast-specific MAP that plays several roles in regulating the yeast microtubule network. Previous work from the authors and other labs has established that She1 binds to microtubules and negatively regulates dynein function to promote mitotic spindle positioning. The main goal of this study is to test the hypothesis that She1 acts to polarize dynein pulling forces toward the daughter cell. This builds from previous in vitro reconstitution studies by the authors.

Overall, this study presents a large amount of data investigating She1 function through the use of various approaches, particularly live-cell imaging. The evidence that She1 regulates dynein activity is not necessarily a new finding, but the results here give a very close look at the nature of the spindle movement phenotype in she1 mutants using carefully designed, quantitative microscopy assays. The authors also use a clever array of mutants to test predictions related to She1 function. This is an impressive amount of work. However, the results taken together do not present clear, strong and cohesive support for the conclusions.
The study is also weakened by several major issues regarding experimental design and apparent conflicts with previously published results related to the asymmetric localization of dynein in yeast and the polarization of microtubules towards the daughter cell. These major issues are elaborated below. Addressing these would strengthen the study and support a more convincing model for She1 function.

Comments for the author

Major points
1) The data presented in the study are often very dense, in a way that I found difficult to follow. In particular, Figures 4-6 include multiple dot plots comparing different features of spindle movement across many mutant strains. The text describes these results with simple conclusions, but the data in the figure is much more difficult to interpret. In many cases of the differences between these distributions appear to be quite subtle and it is difficult to make comparisons across the many different mutants. Several of these mutants are not important for the central conclusions of the study, and could be moved to supplemental figures to help clarify these main figures. For example, the rationale for using the CAP-Gly truncation mutant is not particularly strong, and the results for the CAP-Gly mutant strains are largely negative. The results from the She1-NLS mutant might also be moved to a supplemental figure; see minor point #9 below. Moving that data to a supplemental figure could help focus these main figures without detracting from the conclusions.

2) The authors propose a model where She1 inhibits dynein activity in the mother cell by blocking the offloading step. This model predicts that in wild-type cells inactive dynein should localize to astral microtubule ends in the mother cell, and perhaps be enriched on astral microtubules in the mother cell due to a failure to offload. However, previous work has established that dynein does not localize to astral microtubules in the mother cell. Instead, dynein is asymmetrically recruited to astral microtubules that emanate from the daughter-proximal SPB and extend into the bud. Recent work from the Barral lab shows that this asymmetry depends on signals from the daughter-proximal SPB and the kinesin Kip2. The authors do not describe these results, but do state that "a greater number of dynein foci are apparent in mother cells, likely due to their age-dependent accumulation" (page 4). This may be misleading to some readers. My understanding is that this population of dynein foci is on the mother cell cortex and is thought to be inactive. This should be made clear for readers who are not steeped in the yeast dynein lore, and the appropriate papers should be cited.

It seems difficult to reconcile the previously published evidence of dynein asymmetry with the model proposed here, which implies that dynein localizes to the ends of astral microtubules emanating from both SPBs. If dynein is rarely found at the ends of astral microtubules in mother cells, then She1-mediated inhibition of dynein on those microtubules would seem to be a minor feature of dynein regulation. The authors should address this discrepancy and describe how the results from the Barral lab impact their model for She1 function.

3) The asymmetry model presented here relies on the conclusion that She1 preferentially associates with astral microtubules in the mother cell. There are several major flaws in the experiments presented in Figure 7 that weaken this conclusion. First, Figure 7 does not image native levels of She1, but instead images She1 that is overexpressed from a galactose-inducible promoter. This is unlikely to represent the localization and dynamics of the native She1 protein. In fact, She1 is known to be toxic when overexpressed, so it must be assumed that the overexpressed protein has aberrant function and the localization of overexpressed She1 may not represent the situation at native levels. Second, the images shown in Figure 7A appear to show She1-3GFP signal that is limited to the nucleus. Most of the signal in these images is localized to spindle microtubules, but there is also a dim region of signal around the spindle. The size and position of this region are consistent with the nucleus, and She1 is known to localize to the nucleus.
This is problematic because the microtubules that are noted with arrowheads in Figure 7A appear to be exactly within this region, raising the possibility that these are nuclear microtubules rather than microtubules in the mother cytoplasm.
To address this possibility the imaging experiments could be repeated in cells that also express a label for the nucleus, such as the constructs used in Figure 3. The analysis could then focus only on astral microtubules that do not overlap with the nucleus, eliminating the possibility that the experiment is measuring nuclear She1. A second possibility would be to add a strong nuclear export signal to She1 to drive it out of the nucleus. This might make it easier to measure cytoplasmic localization.

The nocodazole experiment in Figure 7E raises similar concerns. If most of the She1 signal is localized to the nucleus, and the nucleus is typically located in the mother in the S-phase arrested cells, then fluorescence measurements within the mother are more likely to include signal from She1 in the nucleus. In addition, the slight bias of signal in mother vs daughter that is shown in 7E could simply emerge from the larger volume of the mother, if full Z volumes were used for this experiment (this is not clear in the manuscript). A control of cytoplasmic GFP would be useful here.

4) Another major concern is the use of the kar9Δ null mutant, which could create artifacts in many of the experiments by disrupting normal astral microtubule polarity. KAR9 is known to polarize the yeast microtubule network toward the bud.
Many (all?) of the spindle movement assays in cells use strains that carry the kar9Δ null allele for the purpose of disrupting Kar9-dependent spindle positioning and isolating dynein-dependent spindle movement. This is mentioned sparsely in the manuscript, but is clear from the figure legends and the strain table. This is a concern because kar9Δ mutant cells disrupt normal microtubule orientation towards the daughter and also exhibit longer microtubules from the mother SPB. Both of these effects are likely to contribute to the changes in the polarity of spindle movement reported here for she1Δ kar9Δ double mutants, making it difficult to disentangle the

contributions of either mutation. The authors should address this by performing spindle movement assays in Figures 4, 5 and 6 with she1Δ single mutants that have normal KAR9 function. This would be more representative of 'normal' microtubule polarity, and isolate the effect of the she1Δ mutant.

5) Figure 5 is difficult to interpret. The dynein mMTBD mutants and the she1Δ mutant phenotypes are clearly different, and the combinations show synergistic effects. It seems that the high and low microtubule-binding affinity of the mMTBD constructs must have other effects besides altering She1 binding and activity.
This makes it difficult to interpret these results.

6) Page 19, paragraph 2. "This revealed that the number of cortical contacts was significantly greater in daughter than mother cells (Fig. 6B), consistent with previous observations that the daughter-oriented SPB nucleates more and longer microtubules50-53. Deletion of She1 had very little impact on the total number of cortical contacts in either mother or daughter cells (Fig. 6B)."
There are two issues with this conclusion. First, the data in Figure 6B supports the opposite conclusion. Contact frequencies shown here are higher in the mother than the daughter for every genotype tested. This is a surprising result because astral microtubules are normally emanating from the daughter-proximal SPB and extend into the bud to contact the bud cortex. The finding here that the astral microtubules more often contact the mother cortex could be attributable to the use of the kar9Δ allele in all of these strains.
The second issue is that she1Δ mutant data in Figure 6B shows a higher ratio of mother:daughter microtubule contacts than the wild-type control. The text quoted above focuses on comparing the frequency of mother or daughter contacts in wt vs she1Δ. Those comparisons may not reveal a significant difference (though the p-values are quite small) but the more informative comparison may be the ratio of mother:daughter contacts for each genotype. There appears to be a strong effect on this ratio for she1Δ, raising the possibility that She1 may regulate the frequency of microtubule contacts with the cell cortex.
On a related note, in Figure 6D the data for wt shows an equal fraction of productive sliding events in the mother and daughter. That is surprising, since the model is that dynein activity is inhibited in the mother by She1. Shouldn't wt cells show more activity towards the daughter?

7) She1 has been previously shown by the Barnes lab and by the authors to inhibit the recruitment of the dynactin complex to astral microtubule ends. The mechanism of regulation is not clear. Since dynactin is required for dynein-dependent spindle movement in yeast, it stands to reason that unregulated dynactin recruitment to microtubule ends could contribute to the increased and aberrant spindle movement seen in she1Δ mutants. Dynactin recruitment is not tested in this manuscript. To my knowledge, dynactin recruitment does not require the CAP-Gly domain of Nip100, so the rationale for testing CAP-Gly truncation alleles here is not strong and do not settle the issue.

8) Page 6, paragraph 1; and pages 25-26. The authors report that she1Δ cells with mispositioned spindles exhibit similar anaphase durations as she1Δ cells with properly positioned spindles, and conclude that She1 may be important for both spindle positioning and the Spindle Position Checkpoint. To make this conclusion it is important to know whether the spindles in she1Δ mutant cells move into the daughter before exiting mitosis, and whether she1Δ mutants exhibit an elevated frequency of binucleate cells that exit mitosis while the spindle is still in the mother. Even transient spindle movement into the bud can satisfy the SPC and trigger mitotic exit, and she1Δ mutants appear to be defective at maintaining the spindle in the bud neck so they might be expected to show transient excursions.
This is an important point because simultaneous disruption of spindle positioning and the checkpoint (e.g. combining a dyn1Δ mutation with a bfa1Δ mutation) is known to produce a severe negative genetic interaction. If the she1Δ single mutant elicits both effects simultaneously, then I would expect the she1Δ single mutant to exhibit a severe growth defect and perhaps be lethal. But this does not seem to be the case. The authors should clarify this point and amend the discussion accordingly.

Minor
1) Most of the analysis appears to use 2 technical replicates. The typical standard is 3 technical replicates.

2) Figure 1A. What are the genotypes of the cells shown here? Why is the mTurquoise2-Tub1 signal so dim in the bottom cell?

3) Figure 1C-F. Other roles for She1 have been reported, including roles in regulating the stability and disassembly of the mitotic spindle. These roles in the spindle appear to be dynein-independent, but should be considered here since they could impact observations of spindle assembly and anaphase duration?

4) The colors used in Figure 2 B-E are difficult to interpret for colorblind people.

5) Figure 2E. I'm struggling to understand the effect of She1 on stall time. The results state that the stall time increases "4.6 fold". In the figure, 2nM She1 appears to have little effect on stall time, and 5nM She1 appears to increase stall time, but only for a minority of events. Figure 1E shows an odd distribution for 5nM She1, with only ~30% of events exhibiting stall times >100sec. Is this bimodal? Are there two populations of stall events in 5nM She1?

6) Figure 3. It is not clear that this experiment is effectively measuring nuclear translocation at a level sensitive enough to detect defects. The results do not reveal any differences for she1Δ vs wt controls, nor do they show a clear effect for the bni1Δ mutant, which is presented as a positive control. Or perhaps I'm misinterpreting this result? The distribution of datapoints in 3D are almost all clustered at 1.0, 0.5 or 0.0. This might be better presented as categorical data with a chi-squared test. Regardless, without establishing the sensitivity of the experiment, this figure is not informative and we cannot draw conclusions about the impact of she1Δ on nuclear translocation.

7) Figure 3C. What are the genotypes of the cells shown here?

8) Figure 4D. The results here indicate that the frequency of dynein-dependent spindle movements are increased in sheΔ mutants. This seems inconsistent with previous results from the authors (Figure 7D in Ecklund et al 2017) which showed a similar frequency of dynein-dependent microtubule sliding events in she1Δ mutants and wt controls. Why do the experiments in the current manuscript now suggest a role for She1 in attenuating the initiation of sliding events?

9) The she1-NLS allele is intended to drive She1 into the nucleus and deplete it from the cytoplasm. However, the manuscript does not include data confirming this effect; i.e. loss of She1 from the cytoplasm. Without that data, it is difficult to tell whether this manipulation is having the intended effect.

10) Similarly, the G437R mutant in tub1 is difficult to interpret. Figures 4B, C and E show stronger phenotypes for the she1Δ G437R double mutant than for she1Δ single mutants, suggesting the effect of G437R extends beyond a partial disruption of She1 binding to microtubules.

11) Figure 4G. I am very confused by this plot. Does each measurement have two dots per genotype; e.g. if a cell has a spindle that spent 20% of the time in the bud, then it would have a dot at 20% on the daughter side of the plot and 80% on the mother side? If so, why are there dots at 100% mother but not at 0% daughter?

Was the statistical analysis just performed on mother data or bud data? There must be a clearer way to present this data. Maybe 4G could be omitted since 4H is sufficient and much more clear and intuitive?

12) Page 12. "Given the biased residence of the spindle within she1Δ mother cells, these data suggest that She1 may specifically attenuate dynein activity within the mother cell by an unknown mechanism." But Figure 4G and H show that the spindles in she1Δdyn1Δ cells are even more biased to reside in the mother than she1Δ single mutants. This indicates that dynein is not necessary for the mother-cell bias in she1Δ.

13) Figure 7F. Does She1 inhibit dynein stepping/initiation, or dynein-dynactin interaction, or offloading, or some combination? The study tests several possibilities, but the model here does not clearly show the likely mechanism.

Reviewer 3

Advance summary and potential significance to field

In their manuscript, Ecklund et al. investigate how the microtubule-associated protein (MAP), She1, affects dynein-mediated movement and subsequent positioning of the mitotic spindle towards the daughter cell during budding yeast cell division. Prior in vivo work from this group has shown that She1 plays a role in polarizing this movement toward the daughter cell, but the underlying mechanism has not yet been defined.
In addition, this group has previously shown that She1 reduces dynein velocity and dissociation rate using elegant in vitro assays with purified proteins. The in vivo implications of this MAP's clear effect on dynein are now fully explored in this manuscript. The authors perform time-lapse imaging of yeast cells in a variety of mutant backgrounds over multiple cell cycles to capture the movements and positioning of spindle pole bodies, the nucleus and microtubules at various stages of the cell cycle. They find that due to the preferential binding of She1 to astral microtubules within the mother cell, She1 is able to inhibit the initiation of dynein-mediated spindle movements in the mother cell. However, the lack of She1 in the daughter cell therefore allows for the initiation of dynein-mediated spindle movement towards the daughter cell. This is an impressive paper with a plethora of data. The experiments are sound, well-controlled and performed with rigor. The data and statistical analyses are well presented as well. Overall, the authors have thoroughly deconstructed this process and provide a sophisticated and logical model based on their data, and this paper should be accepted at J. Cell Sci.

Comments for the author

The number of experiments and genetic conditions can feel a tad overwhelming while trying to understand what each piece of data plays in the final model. The reader would benefit from having the model explained a bit better in that final paragraph of the introduction (i.e. a brief synopsis of the more thorough explanation on page 24), specifically spelling out the differences in the mother vs. daughter cell. This will help the non-expert reader substantially before they delve into the genetic experiments. Also, a final summary sentence included at the end of each results section would also help keep the reader engaged. Finally, the force results are very interesting, both the in vitro measurements and the in vivo experiments testing the hypothesis that She1 induces a persistent force state of dynein. The experiments with the BNI1 deletion are especially clever but due to the fact that the authors then rule out the assisted force model, perhaps the authors could think about moving some of this data to the supplement to streamline the findings that support their final model.
This is entirely up to the authors however. After a few textual changes, I fully support publication of this work.

First revision

Author response to reviewers' comments

Summary of Changes to Ecklund *et al*. Manuscript

Changes to the manuscript
We have made extensive changes throughout the text to reflect new data (as described in detail below), and in response to comments from the referees. All significant and relevant changes are highlighted in yellow throughout the main and supplemental text.

Changes to the Figures
We have added new data to provide additional support for our proposed model, and also in response to valuable suggestions by the referees.

Figure 1	We have added genotype labels to the cell images in panel A in response to a comment by referee #2.

Figure 2	We have changed the colors used in this figure to those more discernible by the colorblind, as suggested by referee #2.
Figure 3	We have added new data, as shown in panels E – G, which describes the quality of dynein-mediated nuclear migration in various strains. These were added in response to comments made by referee #2.
Figure 4	We have removed data pertaining to the *nip100$^{\Delta CAP\text{-}gly}$* mutant from this figure, as suggested by referee #2. These data are now presented in Figure S4.
Figures 5 and 6	We have added new data to these figures describing the behavior of spindle movements in cells expressing a dynein mutant that is less able to adopt the autoinhibited 'phi' conformation. These were added in response to comments by referee #1.
Figure 7	This is a new figure in which we describe a quantitative analysis of dynein and dynactin localization in various strains. These data were added in response to comments by referee #2.
Figure 8	This figure (which was originally Figure 7) has new data in which we present additional evidence for the asymmetric localization of She1 to astral microtubules within the mother cell. These data were added in response to comments by referee #2.
Supplementary Figure 1	This figure is unchanged.
Supplementary Figure 2	This figure (which was originally Figure S3) includes new data describing a quantitative analysis of spindle movements in cells with wild-type *KAR9*. These data were added in response to comments by referee #2.
Supplementary Figure 3	This figure (which was originally Figure S2) includes new data describing the localization of She1^{NLS} in cells, and the consequences of overexpressing this protein on cell growth.
Supplementary Figure 4	This is a new figure in which all of the data pertaining to the *nip100$^{\Delta CAP\text{-}gly}$* mutant is now situated, as suggested by referee #2.
Supplementary Figure 5	This figure presents additional evidence for the asymmetric localization of She1 to astral microtubules within the mother cell. These data were added in response to comments by referee #2.

Response to Referees

We would like to thank all the referees for their valuable comments and criticisms, and the very thoughtful commentary that helped improve our manuscript. We know a significant amount of effort is required to read and review a manuscript (especially one with as much data as this one), and to provide insight and helpful commentary, and we very much appreciate it.

Detailed Response to Referee #1

1. Most of the experiments were done in the kar9Δ background, and do you see the oscillatory spindle movements across the neck as first described by Yeh et al., 2000 MBoC? If so, it would be better to mention that. I think that in the Moore et al., 2009 PNAS study, the authors also used the kar9Δ background to detect a function of p150 GAP-Gly. On page 15, you wrote that "*our analysis of spindle movements in nip100ΔCAP-gly mutant cells revealed no significant impact on dynein*

function (Fig. 4A – H). These data contrast with prior observations[38]*.*" Did you use the kar9Δ background for the experiments described in Fig. 4? It would be better to clarify this point and modify the conclusion if necessary.

Much like the study by Moore *et al.* (2009 PNAS), we did indeed perform these analyses in a *kar9Δ* mutant background. We have clarified this point in the revised manuscript. It is also worth mentioning that our revised manuscript now includes additional data in which we have quantitated spindle movements in cells expressing wild-type *KAR9* (as suggested by referee #2). We thank the reviewer for the suggestion.

2. Page 25, "*Thus, the She1-dynein MTBD contacts may directly promote the autoinhibited conformation, which is mediated in part by contact points in the coiled-coil stalk within the heavy chain*[46,54]*,*" This is an interesting point although speculative. I would suggest adding a paper by "Niekamp et al., 2019 EMBO" here to say that changes in the stalk can indeed be transmitted to the motor ring. Potentially, you could check if the dynein-LIS1 interaction is affected by She1 and if introducing the phi mutation that keeps dynein open would make a difference. While these experiments are not essential for the main conclusion of the current paper, a more detailed discussion on this point, including the auto-inhibited phi vs. open conformation of dynein and LIS1's role in promoting the open state as revealed recently in yeast, Aspergillus, in vitro, should help the reader understand this point more easily. In addition, could it also be possible that dynein-dynactin simply fall apart during offloading if dynein gets stuck on the microtubule for too long?

We thank the reviewer for suggesting the Niekamp *et al.* paper. Our revised manuscript includes this citation along with a couple others that are relevant to support this point (Uchimura *et al.*, JCB 2015; and, Kon *et al.*, 2009 Nat Struct Mol Biol).

Although we think testing whether She1 affects the dynein-LIS1 interaction is a great idea in principle (as a metric to determine whether She1 affects the phi conformation). However, this experiment is technically challenging in light of the fact that microtubules are likely required for this effect. As an alternative, we are including new data in our revised manuscript that we collected by analyzing spindle dynamics in a dynein mutant that we think is compromised in its capacity to adopt the phi conformation (*dyn1*^{D2868K}). Interestingly, these new data (see new Figures 5 and 6) indeed support the notion that She1 may in fact function by promoting the phi conformation, as this mutant is somewhat refractory to She1 activity in cells. Please find discussion about these new results in our revised manuscript.

Finally, although we cannot exclude the hypothesis that dynein-dynactin fall apart during the offloading process if dynein remains stuck to the microtubule for too long, we prefer the model in which She1 simply promotes the phi conformation. We thank the reviewer for the great suggestion and thoughtful comments.

3. Here I would make several minor suggestions on the text:
(1) Abstract, "dynein microtubule-binding domain, and to astral microtubules", the comma can be removed. It would also be better to say "and its preferential binding to astral…".

We thank the reviewer for the editorial suggestions, both of which have been made.

4. Page 4, "orthologous to NuMA in humans". You may consider adding "Greenberg et al., 2018, Biophys Rev" as a reference.

We have added the citation as suggested. We thank the reviewer for the suggestion.

5. Page 4, "*a greater number of dynein foci are apparent in mother cells*," Please add a reference.

We have never quantitated this phenomenon (nor do we know of anyone that has), but always assumed this was the case (based on qualitative assessment of live cell movies). Our new analysis in fact reveals this is not the case (see new Figure 7B and C). We thank the reviewer for indirectly asking for this information.

6. Page 6, "*mispositioned spindles trigger a spindle positioning checkpoint (SPC)*," you may consider adding "Yeh et al., 1995 JCB" as an original reference.

We have added the citation as suggested. We thank the reviewer for the suggestion.

7. Page 8 "*KAR9, a key member of an actomyosin-mediated spindle orientation pathway*[37]." You may consider adding "Miller and Rose 1998 JCB" as an original reference.

We have added the citation as suggested. We thank the reviewer for the suggestion.

8. Page 13 "spindle microtubule stability[43]," should be changed to "spindle stability[43]".

We have made the suggested change to the text. We thank the reviewer for the suggestion.

Detailed Response to Referee #2

Major Points:

1. The data presented in the study are often very dense, in a way that I found difficult to follow. In particular, Figures 4-6 include multiple dot plots comparing different features of spindle movement across many mutant strains. The text describes these results with simple conclusions, but the data in the figure is much more difficult to interpret. In many cases of the differences between these distributions appear to be quite subtle and it is difficult to make comparisons across the many different mutants. Several of these mutants are not important for the central conclusions of the study, and could be moved to supplemental figures to help clarify these main figures. For example, the rationale for using the CAP-Gly truncation mutant is not particularly strong, and the results for the CAP-Gly mutant strains are largely negative. The results from the She1-NLS mutant might also be moved to a supplemental figure; see minor point #9 below. Moving that data to a supplemental figure could help focus these main figures without detracting from the conclusions.

We agree that our findings from the CAP-gly truncation data are not particularly illuminating of any mechanistic basis by which She1 affects dynein function. We have thus moved these data to a supplemental figure (see new Figure S4). However, in spite of the concerns raised by the reviewer (see Minor point #9 below), we think the She1^{NLS} data are in fact important enough to keep in the main figure. We thank the reviewer for the comments.

2a. The authors propose a model where She1 inhibits dynein activity in the mother cell by blocking the offloading step. This model predicts that in wild-type cells, inactive dynein should localize to astral microtubule ends in the mother cell, and perhaps be enriched on astral microtubules in the mother cell due to a failure to offload. However, previous work has established that dynein does not localize to astral microtubules in the mother cell. Instead, dynein is asymmetrically recruited to astral microtubules that emanate from the daughter-proximal SPB and extend into the bud. Recent work from the Barral lab shows that this asymmetry depends on signals from the daughter-proximal SPB and the kinesin Kip2. The authors do not describe these results, but do state that "*a greater number of dynein foci are apparent in mother cells, likely due to their age-dependent accumulation*" (page 4).
This may be misleading to some readers. My understanding is that this population of dynein foci is on the mother cell cortex and is thought to be inactive. This should be made clear for readers who are not steeped in the yeast dynein lore, and the appropriate papers should be cited. It seems difficult to reconcile the previously published evidence of dynein asymmetry with the model proposed here, which implies that dynein localizes to the ends of astral microtubules emanating from both SPBs. If dynein is rarely found at the ends of astral microtubules in mother cells, then She1-mediated inhibition of dynein on those microtubules would seem to be a minor feature of dynein regulation. The authors should address this discrepancy and describe how the results from the Barral lab impact their model for She1 function.

We apologize for omitting these important citations regarding asymmetric recruitment of dynein to microtubule plus ends in the daughter cell as described by the Barral lab. Our revised manuscript includes this important and highly relevant information.

As noted above, we have now included a new analysis in our revised manuscript in which we measured the relative extent to which dynein (and dynactin) localize to microtubule plus ends and cortex in mother and daughter cells. As noted above (Referee #1, point #5), our original assumption about the asymmetric accumulation of dynein in mother cells was incorrect, and our new results in fact show that cortical foci of dynein are equally apparent in both mother and daughter cells. We have no reason to assume that these cortical foci in the mother cells are any more or less active than those in the daughter cell; in fact, our data show that dynein is indeed active in the mother cell to varying extents in all yeast strains.

Importantly, we note that a significant proportion of wild-type mother cells indeed exhibit dynein and dynactin foci at their plus ends (51% and 32%, respectively); thus, She1-mediated inhibition of dynein in mother cells is likely required to prevent these molecules from moving the spindle in this compartment. Of additional interest, our new results also support a model whereby She1 may function in part by modulating the extent to which dynein localizes to plus ends in a compartment specific manner. Although the mechanism by which She1 may impact dynein or dynactin localization is unclear, it could potentially be related to She1's ability to modulate the phi conformation (which precludes the dynein-Pac1 interaction, which is required for plus end-association; see new Figures 5 and 6).

It is worth noting that in spite of observations noting a lower degree of microtubule plus end localization of dynein in the mother cell (*e.g.*, those from the Barral lab), dynein indeed appears to be quite active in this compartment. The studies from the Barral lab – which are indeed of great interest, and likely represent an important mechanism underlying the regulation of dynein localization – focused exclusively on localization, and did not assess activity. A more accurate read-out for its presence is its activity, which is very apparent in our assays. We thank the reviewer for reminding us about these important data, and for the important questions that forced us to revise our manuscript.

3. The asymmetry model presented here relies on the conclusion that She1 preferentially associates with astral microtubules in the mother cell. There are several major flaws in the experiments presented in Figure 7 that weaken this conclusion. First, Figure 7 does not image native levels of She1, but instead images She1 that is overexpressed from a galactose-inducible promoter. This is unlikely to represent the localization and dynamics of the native She1 protein. In fact, She1 is known to be toxic when overexpressed, so it must be assumed that the overexpressed protein has aberrant function and the localization of overexpressed She1 may not represent the situation at native levels. Second, the images shown in Figure 7A appear to show She1-3GFP signal that is limited to the nucleus. Most of the signal in these images is localized to spindle microtubules, but there is also a dim region of signal around the spindle. The size and position of this region are consistent with the nucleus, and She1 is known to localize to the nucleus. This is problematic because the microtubules that are noted with arrowheads in Figure 7A appear to be exactly within this region, raising the possibility that these are nuclear microtubules rather than microtubules in the mother cytoplasm. To address this possibility the imaging experiments could be repeated in cells that also express a label for the nucleus, such as the constructs used in Figure 3. The analysis could then focus only on astral microtubules that do not overlap with the nucleus, eliminating the possibility that the experiment is measuring nuclear She1. A second possibility would be to add a strong nuclear export signal
to She1 to drive it out of the nucleus. This might make it easier to measure cytoplasmic localization. The nocodazole experiment in Figure 7E raises similar concerns. If most of the She1 signal is localized to the nucleus, and the nucleus is typically located in the mother in the S-phase arrested cells, then fluorescence measurements within the mother are more likely to include signal from She1 in the nucleus. In addition, the slight bias of signal in mother vs daughter that is shown in 7E could simply emerge from the larger volume of the mother, if full Z volumes were used for this experiment (this is not clear in the manuscript). A control of cytoplasmic GFP would be useful here.

Although the concern stated by the reviewer regarding overexpressed She1 is indeed a valid one, we have no reason to suspect that the overexpressed protein has aberrant function, but is simply rather more concentrated in the cells. For example, we know that purified overexpressed She1 (overexpressed for 3 hours in galactose-containing media) binds microtubules *in vitro* with expected binding affinity (Denarier et al., MBoC 2021), and interacts with dynein on these microtubules (Ecklund et al., unpublished). Our *in vivo* data also show that overexpressed She1 binds both microtubules and dynein in cells (see Figure 8F and G, and Figure S5C and D). It is also

worth noting that the toxicity due to She1 overexpression can be eliminated when the protein is concentrated in the nucleus (see *GAL1p:she1*^{NLS} growth phenotype in Fig. S3G), thus indicating that the cell cycle arrest is a consequence of cytoplasmic accumulation of She1, and not simply a consequence of She1 overexpression. With regard to the referee's concern that overexpressed She1 is unlikely to represent the localization of native protein: it seems that overexpression would be more likely to disrupt an asymmetric localization pattern than to be responsible for one. In fact, given the overexpressed protein retains an asymmetric localization pattern, this indicates that the mechanisms underlying the asymmetry are sufficiently robust to operate even with higher than normal protein levels.

The reviewer has also expressed a valid concern about whether the She1 fluorescence data presented in the previous Figure 7 (now Figure 8) was cytoplasmic or nuclear, and questioned the identity of the dim She1 fluorescence in the vicinity of the spindle. We have spent a considerable amount of effort in the last few months trying to determine whether the She1-3GFP fluorescence signal is present on cytoplasmic or nuclear microtubules, and to determine where these dim 'clouds' of She1-3GFP fluorescence are situated (see Figure 8A, blue dashed circle for an example). In particular, we have attempted imaging the nucleus (NLS-3mCherry) and overexpressed She1-3GFP at the same time (as suggested by the reviewer), which was unfortunately inconclusive due to ambiguities in distinguishing nuclear from cytoplasmic microtubules (largely a consequence of their short length, as shown in Figure 8A).

For these reasons, we turned to two alternative methods to determine in which compartment (mother versus daughter) the microtubule-bound She1 resides, and the nature of the diffuse She1 'clouds'. In both, we are exploiting two well established phenomenona: (1) dynein is localized in the cytoplasm, and is excluded from the nucleus (note the absence of dynein fluorescence from the spindle regions in Figure 8F and S5C); and, (2) She1 overexpression leads to a relocalization of dynein from microtubule plus ends to along the length of astral microtubules in a manner that requires dynein's microtubule binding domain (Ecklund et al., Nat Comm 2017). We reasoned that assessment of dynein localization (*i.e.*, to mother versus daughter microtubules) in She1-overexpressing cells would reveal where She1 is localizing.

In the first method, we assessed Dyn1-3YFP localization in cells overexpressing unlabeled She1. For reasons that are unclear (although likely a consequence of the mispositioned anaphase spindles, which tend to have long astral microtubules), microtubules in these cells are longer than those in non-She1-overexpressing cells (note that unlike those cells in Figure 8A-E, these cells were not HU-arrested), thus providing us with several examples of microtubules that span from the mother to the daughter compartment. These images revealed numerous examples of cells with Dyn1-3YFP bound to the length of astral microtubule that is within the mother, but not that within the daughter.
Example images of this are now included in Figure 8F and S5C.

In the second method, we assessed Dyn1-3mCherry localization in cells overexpressing She1-3GFP and unlabeled Kip2. Overexpression of Kip2 has been shown to lead to a large increase in the length of astral microtubules specifically (Carvalho et al., 2004). Thus, colocalizing Dyn1-3mCherry and She1-3GFP on long microtubules would be indicative of astral microtubule She1 localization, specifically. As shown in our new Figures 8G and S5D, this is indeed what we observed; specifically, we noted colocalization of She1 and Dyn1 along long microtubules, indicative of She1 localizing to astral microtubules. Note that overexpression of *KIP2* alone does not lead to astral microtubule decoration by dynein (Lammers *et al.*, JCB 2015).

These latter images also revealed the nature of the diffuse She1-3GFP 'clouds'. In particular, we noted that these dim fluorescence clouds corresponded to regions within the cytoplasm, as is apparent from their overlapping with dynein-bound, very long (due to Kip2 overexpression) astral microtubules.

As a result of these new findings, we are now confident that the She1 localization pattern quantitated in Figure 8B – D is indeed indicative of astral microtubule-bound She1. This is consistent with the fact that the microtubules on which She1 are apparent (*e.g.*, Fig. 8A) are emanating out of one side of the spindle, where the SPB is situated. Given that SPBs are embedded in the nuclear envelope, it seems unlikely that these microtubules are nuclear given their position with respect to the pole.

Finally, in answer to the reviewer's suggestion, we also attempted to generate a She1^{NES} construct (with nuclear export signal), but it didn't appear to be sufficient to exclude She1 from the nucleus (it exhibited a localization pattern indistinguishable from wild-type She1). We thank the reviewer for raising very valid concerns that have led to experiments that we feel have

significantly strengthened our conclusions.

4. Another major concern is the use of the kar9Δ null mutant, which could create artifacts in many of the experiments by disrupting normal astral microtubule polarity. KAR9 is known to polarize the yeast microtubule network toward the bud. Many (all?) of the spindle movement assays in cells use strains that carry the kar9Δ null allele for the purpose of disrupting Kar9-dependent spindle positioning and isolating dynein-dependent spindle movement. This is mentioned sparsely in the manuscript, but is clear from the figure legends and the strain table. This is a concern because kar9Δ mutant cells disrupt normal microtubule orientation towards the daughter and also exhibit longer microtubules from the mother SPB. Both of these effects are likely to contribute to the changes in the polarity of spindle movement reported here for she1Δ kar9Δ double mutants, making it difficult to disentangle the contributions of either mutation. The authors should address this by performing spindle movement assays in Figures 4, 5 and 6 with she1Δ single mutants that have normal KAR9 function. This would be more representative of 'normal' microtubule polarity, and isolate the effect of the she1Δ mutant.

The reviewer is correct that we performed all of our spindle dynamics assays in the absence of *KAR9*. Also as stated by the reviewer, the purpose of this was to isolate dynein-dependent spindle movements from those that may be mediated by the *KAR9* pathway. We respectfully disagree with the reviewer that repeating all of our analyses in a wild-type *KAR9* background is necessary to appropriately discern the role of She1 in the polarization of dynein-dependent spindle movements.

Our reasons for this are four-fold: (1) by comparing results between *kar9Δ SHE1* and *kar9Δ she1Δ* cells, we are able to isolate the specific contribution of She1 to the baseline spindle movements noted in *kar9Δ* cells, thus permitting us to draw conclusions on the role of She1 in dynein activity (irrespective of *KAR9*); (2) although Kar9 indeed plays a role in the fate of SPB inheritance, the Barral lab noted that Kar9 plays little to no role in affecting the asymmetric distribution of dynein to microtubule plus ends (Grava *et al.*, Dev Cell 2006); (3) our model is built upon data collected from hand curated dynein-mediated spindle movements, such as those described in Figures 4B – E, Figures 5B – E, and Figure 6. These events could only be unambiguously attributed to dynein if all other known force generators have been eliminated (*i.e.*, *kar9Δ*). Addition of *KAR9* to our genetic background complicates this possibility, and would make our analysis and interpretation of our data more difficult. (4) In support of the notion that *KAR9* has no impact on the She1-mediated polarization of dynein activity in cells, we previously reported that the position of the mitotic spindle at the moment of anaphase onset is indeed significantly offset from the bud neck within the mother cell in the presence of a wild-type allele of *KAR9* (see Figure 6H in Markus *et al.*, Curr Biol 2012).

That being said, in order to determine whether the spindle position data are a consequence of deletion of She1 in a *kar9*Δ background, we have now included two additional yeast strains to our analysis: *KAR9 SHE1* and *KAR9 she1Δ*. These new data (in our new Figure S2) indicate that the *she1Δ*-mediated spindle position data noted in *kar9*Δ cells is almost identical in cells expressing Kar9. Thus, She1 polarizes dynein-mediated spindle movements in a manner that is unrelated to Kar9- mediated spindle and/or SPB orientation. We thank the reviewer for the suggestion, which we think adds an important new data point to our analysis.

5. Figure 5 is difficult to interpret. The dynein mMTBD mutants and the she1Δ mutant phenotypes are clearly different, and the combinations show synergistic effects. It seems that the high and low microtubule-binding affinity of the mMTBD constructs must have other effects besides altering She1 binding and activity. This makes it difficult to interpret these results.

We agree with the reviewer that the *dyn1*^{mMTBD} and *dyn1*$^{mMTBD\text{-}HA}$ mutants alone lead to alterations in dynein activity and spindle dynamics (as presented in Figure 5). The reasons for this likely relate to the fact that we have altered the affinity of dynein for microtubules with these two mutants. It is for this reason that all of our conclusions relied on directly comparing results from the respective *SHE1* and *she1Δ* backgrounds (*SHE1 dyn1*^{mMTBD} versus *she1Δ dyn1*^{mMTBD}). In this way, we were able to directly assess the changes in spindle movements that arise as a consequence of She1. We think this is a fair manner in which to interpret our results.

6a. Page 19, paragraph 2. "*This revealed that the number of cortical contacts was significantly*

greater in daughter than mother cells (Fig. 6B), consistent with previous observations that the daughter- oriented SPB nucleates more and longer microtubules[50-53]. Deletion of She1 had very little impact on the total number of cortical contacts in either mother or daughter cells (Fig. 6B).”

There are two issues with this conclusion. First, the data in Figure 6B supports the opposite conclusion. Contact frequencies shown here are higher in the mother than the daughter for every genotype tested. This is a surprising result because astral microtubules are normally emanating from the daughter-proximal SPB and extend into the bud to contact the bud cortex. The finding here that the astral microtubules more often contact the mother cortex could be attributable to the use of the kar9Δ allele in all of these strains.

We thank the reviewer for catching this significant error on our part! We have now corrected this mistake by changing the text accordingly. In answer to the reviewer’s second concern, it is certainly possible that the total number of cortical contacts is attributable to deletion of *KAR9*. However, although we are aware of studies reporting on astral microtubule length differences between mother and daughter cells (Estrem et al., 2017; Lengefeld et al., 2018; Vogel et al., 2001; Vogel and Snyder, 2000), we know of no such study that has measured astral microtubule-cortex encounters. It’s worth noting that one study found that deletion of *KAR9* leads to a very similar increase in the lengths of astral microtubules in the mother and daughter cells (from 0.9 µm in *KAR9* to 1.7 µm in *kar9Δ* mother cells; and from 1.1 µm in *KAR9* to 1.9 µm *kar9*Δ daughter cells; Baumgärtner et al., PLoS One 2014), which would be expected to maintain an equal number of contacts in both compartments (similar to what we observe). Note that the astral microtubule-cortex encounters are not just a product of microtubule length, but are also due to spindle movements. If the spindle moves in close proximity to the mother cell cortex (which it does in all yeast strains imaged), then it has a high probability of contacting that cortex. We thank the reviewer for the thoughtful comments.

6b. The second issue is that she1Δ mutant data in Figure 6B shows a higher ratio of mother:daughter microtubule contacts than the wild-type control. The text quoted above focuses on comparing the frequency of mother or daughter contacts in wt vs she1Δ. Those comparisons may not reveal a significant difference (though the p-values are quite small) but the more informative comparison may be the ratio of mother:daughter contacts for each genotype. There appears to be a strong effect on this ratio for she1Δ, raising the possibility that She1 may regulate the frequency of microtubule contacts with the cell cortex.

The reviewer is correct that there is indeed a change in the ratio of mother:daughter cortical contacts (1.27 in *SHE1* versus 1.92 in *she1Δ*). We have including a description of these differences in the revised manuscript. However, it is important to note that we did not observe a similar extent of change in this ratio in *she1*^{NLS} (1.45), *tub1*^{G437R} (1.35), or *tub1*^{G437R} *she1Δ* (1.35) cells, all of which showed an asymmetric enrichment in the fraction of productive events (as shown in Fig. 6D). Thus, although it is possible that changes in microtubule dynamics partly account for the spindle enrichment phenotype in some backgrounds (*e.g.*, *dyn1*^{D2868K}), we don’t think a change in the number of cortical contacts is the underlying cause for spindle enrichment in the mother cell in others. We have modified the text to reflect this point. We thank the reviewer for the thoughtful comments.

6c. On a related note, in Figure 6D the data for wt shows an equal fraction of productive sliding events in the mother and daughter. That is surprising, since the model is that dynein activity is inhibited in the mother by She1. Shouldn’t wt cells show more activity towards the daughter?

Our data indeed indicate a roughly equal fraction of productive events in mother and daughter wild-type cells. However, the uneven enrichment of dynein-mediated spindle movement events in the mother cells (with respect to the daughter) upon deletion of She1 suggests that it functions to primarily dampen these events in the mother cell. Thus, the ability of She1 to promote daughter cell-directed spindle movements is likely a consequence of attenuating the baseline activity of dynein in the mother cell, which is apparently quite high.

7. She1 has been previously shown by the Barnes lab and by the authors to inhibit the recruitment

of the dynactin complex to astral microtubule ends. The mechanism of regulation is not clear. Since dynactin is required for dynein-dependent spindle movement in yeast, it stands to reason that unregulated dynactin recruitment to microtubule ends could contribute to the increased and aberrant spindle movement seen in she1Δ mutants. Dynactin recruitment is not tested in this manuscript. To my knowledge, dynactin recruitment does not require the CAP-Gly domain of Nip100, so the rationale for testing CAP-Gly truncation alleles here is not strong and do not settle the issue.

We agree with the reviewer's summation of work from the Barnes lab (and some of our previous work), that She1 may be affecting dynein activity by regulating the dynein-dynactin interaction at plus ends, or during the offloading process. Our reason for employing *nip100*$^{\Delta CAP\text{-}Gly}$ mutant cells is that this is the only available such mutant that disrupts dynactin activity (although our findings may challenge this notion). We reasoned that if the *she1Δ* phenotype is a consequence of enhanced dynein-dynactin binding, then potentially reducing dynactin function may have attenuated this phenotype, which did not end up being the case.

That being said, to more directly address the role of dynactin recruitment, as noted above, we have included additional analyses in our revised manuscript in which we determine the relative degree of plus end-association of dynein and dynactin in several yeast strains (see new Figure 7). These data indicate that both dynein and dynactin localization are impacted by loss of She1, suggesting that She1 may in fact impact dynein activity by affecting its association with the plus end-binding machinery (*e.g.*, Pac1, Bik1) and/or dynactin in a compartment-specific manner. We thank the reviewer for the comments that led to our assessment of dynein-dynactin localization.

8. Page 6, paragraph 1; and pages 25-26. The authors report that she1Δ cells with mispositioned spindles exhibit similar anaphase durations as she1Δ cells with properly positioned spindles, and conclude that She1 may be important for both spindle positioning and the Spindle Position Checkpoint. To make this conclusion, it is important to know whether the spindles in she1Δ mutant cells move into the daughter before exiting mitosis, and whether she1Δ mutants exhibit an elevated frequency of binucleate cells that exit mitosis while the spindle is still in the mother. Even transient spindle movement into the bud can satisfy the SPC and trigger mitotic exit, and she1Δ mutants appear to be defective at maintaining the spindle in the bud neck so they might be expected to show transient excursions. This is an important point because simultaneous disruption of spindle positioning and the checkpoint (e.g. combining a dyn1Δ mutation with a bfa1Δ mutation) is known to produce a severe negative genetic interaction. If the she1Δ single mutant elicits both effects simultaneously, then I would expect the she1Δ single mutant to exhibit a severe growth defect and perhaps be lethal. But this does not seem to be the case. The authors should clarify this point and amend the discussion accordingly.

The reviewer raises another really important point about out conclusions regarding the role of She1 in the SPC. A closer look at our time-lapse movies revealed that in almost all instances in which the anaphase spindle was mispositioned in *she1Δ* cells, the daughter-bound SPB of the mispositioned immediately translocated into the daughter cell following anaphase. Such a rapid motion of the SPB into the daughter cell would likely be sufficient to silence the SPC, and promote mitotic exit. Thus, we have changed our conclusion regarding a potential role for She1 in the SPC, and revised the manuscript accordingly. We thank the reviewer for forcing us to reevaluate our time-lapse movies.

Minor Points:

1. Most of the analysis appears to use 2 technical replicates. The typical standard is 3 technical replicates.

All of our data points are presented in a transparent manner with appropriate t-tests. In light of the reproducible nature of our findings in the independent replicates and the *P* values calculated from our data, we think our data are sufficient to draw the conclusions presented.

2. Figure 1A. What are the genotypes of the cells shown here? Why is the mTurquoise2-Tub1 signal so dim in the bottom cell?

We apologize for this oversight. We have now added appropriate labels to Figure 1A. The brightness of the fluorescent proteins (*e.g.*, mTurquoise2) is inversely proportional to the number of images acquired. The images of the spindle in the bottom (*she1Δ*) cell were acquired later in the course of imaging (which took place over 10 hours), and was thus more photobleached than the top (*SHE1*) cell. The imaging conditions were identical for both strains.

3. Figure 1C-F. Other roles for She1 have been reported, including roles in regulating the stability and disassembly of the mitotic spindle. These roles in the spindle appear to be dynein-independent, but should be considered here since they could impact observations of spindle assembly and anaphase duration?

The reviewer is indeed correct that She1 has been shown to impact spindle disassembly, which could account for the phenotype noted in Figure 1E. We have modified the text to stress that this is likely a dynein-independent function for She1. We thank the reviewer for the comment.

4. The colors used in Figure 2 B-E are difficult to interpret for colorblind people.

We have changed the colors employed in Figure 2 to those that are more easily discernible by colorblind people. We thank the reviewer for pointing this out.

5. Figure 2E. I'm struggling to understand the effect of She1 on stall time. The results state that the stall time increases "4.6 fold". In the figure, 2nM She1 appears to have little effect on stall time, and 5nM She1 appears to increase stall time, but only for a minority of events. Figure 1E shows an odd distribution for 5nM She1, with only ~30% of events exhibiting stall times >100sec. Is this bimodal? Are there two populations of stall events in 5nM She1?

The distribution is not, strictly speaking, bimodal, since it does not have two maxima. However, the reviewer is correct that there seem to be two subpopulations. Upon addition of 5 nM She1, ~30% of the stall events lasted >100 s, whereas without She1 such long-duration events were extremely rare (<1%). An intermediate situation occurred at 2 nM She1, with ~4% of the events falling into the long-duration category, >100 s. The distributions do suggest that the durations of only a subpopulation of stall events are being increased by the addition of She1. Although the reason for this is unclear, it may be a consequence of the non-saturating levels of microtubule decoration that takes place with these concentrations of She1, and the short distances over which the trapping experiments take place (and the probability of encountering sufficient microtubule-bound She1). However, we chose these concentrations to approximate that found in cells (~2 nM; Markus *et al.*, Curr Biol 2012).

6. Figure 3. It is not clear that this experiment is effectively measuring nuclear translocation at a level sensitive enough to detect defects. The results do not reveal any differences for she1Δ vs wt controls, nor do they show a clear effect for the bni1Δ mutant, which is presented as a positive control. Or perhaps I'm misinterpreting this result? The distribution of datapoints in 3D are almost all clustered at 1.0, 0.5 or 0.0. This might be better presented as categorical data with a chi-squared test. Regardless, without establishing the sensitivity of the experiment, this figure is not informative and we cannot draw conclusions about the impact of she1Δ on nuclear translocation.

We agree with the reviewer that our nuclear/neck translocation success assay (Figure 3D) may not be sufficiently sensitive to detect defects in force generation by dynein, or the role of She1 in this process. To address this concern, we performed a more careful and detailed analysis of the dynein- mediated motility during the nuclear/neck translocation. Specifically, we assessed the velocity, translocation distance, and duration of these movements (*i.e.*, in the moments in which the nucleus is translocating the neck; see new Figure 3E-G). Interestingly, this analysis indeed revealed that loss of She1 leads to significantly slower velocities during these movements (and a small reduction in the translocation distance), and that combined deletion of She1 and Bni1 increased both the velocity and translocation distance. This findings suggest that She1 may in fact support dynein-mediated force production during the nuclear/neck translocation. We have modified the text to reflect these new data, including changing the header from "*She1 induces a persistent force generating dynein state in vitro but likely not in vivo*" to "*She1 induces a persistent force generating dynein state in vitro and potentially in vivo*." We thank the reviewer

for the suggestion.

7. Figure 3C. What are the genotypes of the cells shown here?

We apologize for omitting this information. We have added this to our updated Figure 3 legend (this is an example of nuclear transit in a *BNI1 she1Δ* cell). We thank the reviewer for pointing this out.

8. Figure 4D. The results here indicate that the frequency of dynein-dependent spindle movements are increased in sheΔ mutants. This seems inconsistent with previous results from the authors (Figure 7D in Ecklund et al 2017) which showed a similar frequency of dynein-dependent microtubule sliding events in she1Δ mutants and wt controls. Why do the experiments in the current manuscript now suggest a role for She1 in attenuating the initiation of sliding events?

The reviewer is correct that results from our new analysis reveals a significant increase in dynein-mediated spindle movements as a result of *SHE1* deletion, whereas those from our previous analysis revealed no such change. The reason for this discrepancy is likely the manner of measurement (and the person conducting them), which has improved over the intervening years. Specifically, the individual that determined the values presented in the Ecklund et al., 2017 paper (who is different than the person that measured those values in the current manuscript) was not as versed in accurately assessing dynein events from live cell movies. As an illustration of the consistency of our new method of assessment, the values presented in the current manuscript for wild-type (*kar9Δ*) cells is 0.640 events cell^{-1} min^{-1}, which is strikingly similar to those presented in our 2019 manuscript (Marzo *et al.*, eLife), which is 0.646 events cell^{-1} min^{-1}.

9. The she1-NLS allele is intended to drive She1 into the nucleus and deplete it from the cytoplasm. However, the manuscript does not include data confirming this effect; i.e. loss of She1 from the cytoplasm. Without that data, it is difficult to tell whether this manipulation is having the intended effect.

As noted above, our revised manuscript now contains additional data characterizing the *she1^{NLS}* allele. Given the difficulty associated with observing native levels of She1, we generated yeast strains overexpressing mCherry-tagged wild-type or She1^{NLS}. This revealed: (1) that addition of the NLS leads to a large (~4-fold) increase in spindle microtubule-binding by She1 (Fig. S3B; as would be expected if it was confined or enriched in the nucleus); and, (2) that overexpression of She1^{NLS}- mCherry leads to no growth arrest phenotype, in stark contrast to overexpression of wild-type She1- mCherry (Fig. S3G), indicating that the NLS is indeed sufficient to alter She1 cellular behavior, and that binding to cytoplasmic microtubules and not spindle microtubules leads to growth phenotypes. In combination with our data indicating a *she1Δ*-like phenotype for *she1^{NLS}* cells in spindle dynamics behaviors (Fig. 4), our data indicating She1^{NLS} is expressed at similar levels to wild-type She1 (Fig. S3A), and our data indicating no change in the affinity of She1^{NLS} for microtubules *in vitro* (Fig. S3C- F), these data indeed support the notion that She1^{NLS} is sequestered in the nucleus, and excluded from the cytoplasm.

10. Similarly, the G437R mutant in tub1 is difficult to interpret. Figures 4B, C and E show stronger phenotypes for the she1Δ G437R double mutant than for she1Δ single mutants, suggesting the effect of G437R extends beyond a partial disruption of She1 binding to microtubules.

The reviewer is correct that the *tub1^{G437R} she1Δ* cells exhibit some phenotypes that are more pronounced than *TUB1 she1Δ* alone, suggesting this mutant may affect other aspects of microtubule function that interplay with the dynein pathway. As we explain in our revised manuscript, the differences measured in some of the dynein activity metrics (velocity, displacement event^{-1}, and displacement cell^{-1} min^{-1}) may be a consequence of microtubule length differences between *TUB1* and *tub1^{G437R}* cells. Specifically, as noted in our related paper recently published (Denarier *et al.*, MBoC 2021): "*…we noted that the mutant cells exhibited a larger*

fraction of long microtubules (38% of microtubules were ≥ 7μm in tub1^{G437R} cells, versus 10% in wild-type cells; Fig. S3B and C), many of which extended from one cell compartment to the other…" Given the correlation between microtubule length differences and dynein activity (Estrem et al., JCB 2017), such changes would be expected to affect dynein activity. We thank the reviewer for pointing this out.

11. Figure 4G. I am very confused by this plot. Does each measurement have two dots per genotype; e.g. if a cell has a spindle that spent 20% of the time in the bud, then it would have a dot at 20% on the daughter side of the plot and 80% on the mother side? If so, why are there dots at 100% mother but not at 0% daughter? Was the statistical analysis just performed on mother data or bud data? There must be a clearer way to present this data. Maybe 4G could be omitted since 4H is sufficient and much more clear and intuitive?

The reviewer is correct that each measurement from a given cell would be represented by two data points in the plots shown in Figures 4G, 5G, S2F and S4G (one for mother fraction, and one for daughter fraction). Our original draft of these plots eliminated all the 0 points to try to avoid confusion. We know realize it created confusion instead, and for this we apologize. We have now added all the 0 points back to this plot. We thank the reviewer for raising this concern.

12. Page 12. "*Given the biased residence of the spindle within she1Δ mother cells, these data suggest that She1 may specifically attenuate dynein activity within the mother cell by an unknown mechanism*." But Figure 4G and H show that the spindles in she1Δ dyn1Δ cells are even more biased to reside in the mother than she1Δ single mutants. This indicates that dynein is not necessary for the mother-cell bias in she1Δ.

Our data as a whole demonstrate that dynein activity in wild-type (*SHE1*) cells results in the translocation of the spindle into the daughter cell; however, loss of *SHE1* leads to a bias in the mother cell (as noted in the quoted text above). The ability of She1 to promote daughter cell-directed spindle translocation events is a direct consequence of it attenuating dynein activity, which is apparent in cells deleted for *DYN1*, in which the spindle almost never leaves the mother cell. In combination with our findings that *DYN1* is required for the *she1Δ*-mediated increase in spindle translocation activity (Fig. S2H), and our many others in which dynein activity is measured (Fig 4D and E, 5D and E, and 6), we are confident in this model.

13. Figure 7F. Does She1 inhibit dynein stepping/initiation, or dynein-dynactin interaction, or offloading, or some combination? The study tests several possibilities, but the model here does not clearly show the likely mechanism.

In light of our new data (*e.g.*, nuclear/neck translocation velocity, and findings from our spindle dynamics assay with *dyn1^{D2868K}*), we have revised the model presented in our original draft. We now think that the best model that fits our data is that She1 plays at least two roles in affecting dynein function: (1) it precludes interaction between dynein and Pac1/Bik1 and dynein and dynactin (potentially by promoting the autoinhibited 'phi' conformation), thus limiting the localization of dynein to microtubule plus ends and the cell cortex, and consequently attenuating initiation of a dynein- mediated event; and (2) it promotes the interaction of Num1-dynein-dynactin complexes with microtubules during a processive spindle movement event, in a manner that is reflective of our single molecule (Ecklund et al., Nat Comm 2017) and optical trapping (Figure 2) results. For the sake of space, and the complexities associated with She1-mediated dynein function, we have removed the cartoon schematic of this model, but added new text that summarize this model in the Discussion. We thank the reviewer for the question.

Detailed Response to Referee #3

1. The number of experiments and genetic conditions can feel a tad overwhelming while trying to understand what each piece of data plays in the final model. The reader would benefit from having the model explained a bit better in that final paragraph of the introduction (i.e. a brief synopsis of the more thorough explanation on page 24), specifically spelling out the differences in the mother vs. daughter cell. This will help the non-expert reader substantially before they delve into the genetic experiments. Also, a final summary sentence included at the end of each results section

would also help keep the reader engaged.

We agree that the sheer number of plots and mutants in our manuscript can be overwhelming! We have made a significant number of changes to our revised manuscript that we hope improve the clarity of trying to synthesize all this information into a cohesive model.

2. Finally, the force results are very interesting, both the in vitro measurements and the in vivo experiments testing the hypothesis that She1 induces a persistent force state of dynein. The experiments with the BNI1 deletion are especially clever, but due to the fact that the authors then rule out the assisted force model, perhaps the authors could think about moving some of this data to the supplement to streamline the findings that support their final model. This is entirely up to the authors however. After a few textual changes, I fully support publication of this work.

As stated above, we have now included additional analysis of the *in vivo* experiments in which we test the assisted force model. These new data in fact lend support to this model, which can no longer be ruled out. For this reason, we are keeping these data in the main figure. We thank the reviewer for the comment and the support!

Second decision letter

MS ID#: JOCES/2021/258510

MS TITLE: The MAP She1 coordinates directional spindle positioning by spatially restricting dynein activity

AUTHORS: Kari H. Ecklund, Megan E. Bailey, Kelly A. Kossen, Carsten K. Dietvorst, Charles L. Asbury, and Steven M. Markus
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 gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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.

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

The Markus lab has done a tremendous amount of work revising this manuscript and I found this manuscript significantly improved. All my previous comments have been addressed satisfactorily, and I fully support the publication of this paper. I especially like their inclusion of a phi dynein mutant and the data leading to the suggestion that SHE1 may promote a phi state. I also like the fact that they analyzed dynein distribution and activity in mother and daughter cells more carefully during the revision. I am also very impressed by how they have addressed the second reviewer's comments, especially the most important comment on the enrichment of dynein in daughter cells as published by Grava et al., 2006. Their new experimental data have clearly provided new insights, which will be of interest to people in the dynein field and those who work on asymmetrical cell division in general.

Comments for the author

The Markus lab has done a tremendous amount of work revising this manuscript and I found this manuscript significantly improved. All my previous comments have been addressed satisfactorily, and I fully support the publication of this paper. I especially like their inclusion of a phi dynein mutant and the data leading to the suggestion that SHE1 may promote a phi state. I also like the fact that they analyzed dynein distribution and activity in mother and daughter cells more carefully during the revision. I am also very impressed by how they have addressed the second reviewer's comments, especially the most important comment on the enrichment of dynein in daughter cells as published by Grava et al., 2006. Their new experimental data have clearly provided new insights, which will be of interest to people in the dynein field and those who work on asymmetrical cell division in general.
I have only a very minor editorial suggestion: Sheeman et al 2003 (in the ref list already) can be inserted in two places on page 15: beside Lee et al., 2003 in the highlighted new text "...its plus end association, which is mediated by Pac1 (Lee et al., 2003)." and after "...indirectly via Bik1 (Lammers and Markus 2015)."

Reviewer 2

Advance summary and potential significance to field

This study by Ecklund and colleagues addresses these knowledge gaps by investigating the She1 protein, a MAP that regulates dynein activity in budding yeast. She1 is a yeast-specific MAP that plays several roles in regulating the yeast microtubule network. Previous work from the authors and other labs has established that She1 binds to microtubules and negatively regulates dynein function to promote mitotic spindle positioning. The main goal of this study is to test the hypothesis that She1 acts to polarize dynein pulling forces toward the daughter cell. This builds from previous in vitro reconstitution studies by the authors.

We commend the authors for the impressive efforts in this revised manuscript. The revised manuscript addresses many of our concerns and the new results and text strengthen the conclusions.

Comments for the author

We do have one concern related to the interpretation of the new results: the new data comparing spindle movements in KAR9 vs kar9Δ is an important test of whether the kar9Δ mutant might obscure the interpretation of the she1Δ mutant phenotype.
The authors conclude on page 7 that: “Analysis of Kar9-expressing cells revealed a very similar pattern for all spindle position metrics noted above in kar9 cells
(Figure S2E – G), indicating that She1 polarizes dynein activity in a manner that is independent of Kar9.”
The new data in Figure S2 panels A, B and D show that spindle velocity displacement and total displacement are significantly different in she1Δ KAR9 cells compared to she1Δ kar9Δ cells. Therefore, the magnitude of the she1Δ effect on dynein activity does appear to depend on KAR9.

The finding that the kar9Δ mutant exacerbates the impact of the she1Δ mutant on dynein suggests that KAR9 and SHE1 may play complementary roles in regulating dynein, and that experiments measuring dynein activity in kar9Δ she1Δ double mutants reflect the additive loss of both.

Reviewer 3

Advance summary and potential significance to field

The authors have adequately addressed my concerns, and I enthusiastically support publication of this work in JCS.

Comments for the author

The authors have adequately addressed my concerns, and I enthusiastically support publication of this work in JCS.

Second revision

Author response to reviewers' comments

Response to Referees

We would like to once again thank the referees for their valuable comments and criticisms that helped improve our manuscript.

Response to Referee #1

1. I have only a very minor editorial suggestion: Sheeman et al 2003 (in the ref list already) can be inserted in two places on page 15: beside Lee et al., 2003 in the highlighted new text "...its plus end association, which is mediated by Pac1 (Lee et al., 2003)." and after "...indirectly via Bik1 (Lammers and Markus, 2015)."

We have added the Sheeman *et al* citations as suggested. We thank the reviewer for pointing out this oversight.

Response to Referee #2

1. We do have one concern related to the interpretation of the new results: the new data comparing spindle movements in KAR9 vs kar9Δ is an important test of whether the kar9Δ mutant might obscure the interpretation of the she1Δ mutant phenotype. The authors conclude on page 7 that: “Analysis of Kar9-expressing cells revealed a very similar pattern for all spindle position metrics noted above in kar9 cells (Figure S2E – G), indicating that She1 polarizes dynein activity in a manner that is independent of Kar9.”
The new data in Figure S2 panels A, B and D show that spindle velocity, displacement and total displacement are significantly different in she1Δ KAR9 cells compared to she1Δ kar9Δ cells. Therefore, the magnitude of the she1Δ effect on dynein activity does appear to depend on KAR9. The finding that the kar9Δ mutant exacerbates the impact of the she1Δ mutant on dynein suggests that KAR9 and SHE1 may play complementary roles in regulating dynein, and that experiments measuring dynein activity in kar9Δ she1Δ double mutants reflect the additive loss of both.

We agree that our results suggest that Kar9 and She1 may play complementary roles in affecting dynein motility metrics in cells, and have amended our manuscript to reflect this point (see below). That being said, our spindle position metrics indicate that She1 affects the mother/daughter bias in a manner that is completely independent of Kar9 (see Figure S2F and G). To this end, we have modified our manuscript as follows (new text is highlighted):

“Interestingly, whereas She1 reduces spindle displacement values and overall dynein activity in KAR9 cells (Fig. S2B-D) - albeit to a somewhat lesser extent - the presence of She1 had

no effect on spindle velocity in these cells (Fig. S2A), indicating that this specific metric is a consequence of combined deletion of She1 and Kar9, and does not account for the polarized spindle movements promoted by She1. These observations suggest that She1 and Kar9 play complementary roles in affecting dynein motility in cells. However, given the fact that the spindle position metrics were statistically indistinguishable in KAR9 SHE1 and kar9Δ SHE1 cells (Fig. S2F and G; P ≥ 0.2118), and KAR9 she1Δ and kar9Δ she1Δ cells (P ≥ 0.3557), our results indicate that She1 polarizes dynein activity in a manner that is distinct and independent of Kar9 function.

We hope this satisfactorily addresses the reviewer's concern. We thank the reviewer for raising this point.

Third decision letter

MS ID#: JOCES/2021/258510

MS TITLE: The MAP She1 coordinates directional spindle positioning by spatially restricting dynein activity

AUTHORS: Kari H. Ecklund, Megan E. Bailey, Kelly A. Kossen, Carsten K. Dietvorst, Charles L. Asbury, and Steven M. Markus
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.