Modeling a disease-correlated tubulin mutation in budding yeast reveals insight into MAP-mediated dynein function

Eric Denarier, Kari Ecklund, Guillaume Berthier, Adrien Favier, Eileen O'Toole, Sylvie Gory-Fauré, Lisa De Macedo, Christian Delphin, Annie Andrieux, Steven Markus, and Cecile Boscheron

Corresponding author(s): Steven Markus, Colorado State University

**Review Timeline:**

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 2021-05-11 |
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*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.)
RE: Manuscript #E21-05-0237
TITLE: Modeling a disease-correlated tubulin mutation in budding yeast reveals insight into MAP-mediated dynein function

Dear Dr. Markus:

Your manuscript has been seen by two expert referees, whose comments are appended below. Both referees felt that your findings are interesting, provide new insight and that they are of interest to the MBC readership. As you will see, the major points of criticism of the referees concern some missing clarifying experiments regarding the relative function of dynein/Kar9 pathways in yeast, some technical concerns regarding the She1 in vitro experiments, concerns that some parts of the manuscript are less developed than others and that in some cases the number of repetition of experiments is minimal.

Whereas the comments of reviewer 2 are more of a general/strategic nature (including a suggestion for restructuring the manuscript), reviewer 1 raises very specific points. We do not expect that all concerns raised by reviewer 1 need to be addressed by additional experiments. However, some additional experiment will be required, particularly to address those "major comments" that are directly relevant to the major conclusions of the study and to address the issue of very small data sets. Several other concerns can be addressed by editorial changes/clarifications/reorganization of the presentation.

We look forward to receiving your revised manuscript, together with a letter indicating the changes you have made and a point-by-point reply to the referees' concerns.

Sincerely,
Thomas Surrey
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Markus,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,
Eric Baker
The current study by Denarier et al. examines the molecular and functional consequences of the alpha-tubulin-G437R mutation. This mutation, when present in the alpha-tubulin isotype TUBA1A causes malformations in cortical development (MCD) in humans. Here, the authors model this mutation, which occurs in a highly conserved region and residue of alpha-tubulin, in the much simpler budding yeast cell. This facilitates examination of the structure/function relationship of the G437R mutation, and the authors take advantage of this to examine the effect on microtubule dynamics and spindle positioning. Notably, they find that G437R disrupts binding the dynein regulatory protein She1, which leads to hyperactive dynein function during spindle positioning. This is overall consistent with findings that MCD-causing mutations perturb dynein function during neuronal migration. In total, the amount of work that has gone into this study, along with the breadth of approaches and techniques, is highly commendable. This study, after suitable revisions, will be a valuable contribution to the fields of microtubule function, tubulinopathies, and tubulin/MAP interactions.

One significant area that needs to be addressed are the assays used to determine the mutations effect on Dynein activity, specifically those used to discriminate between Dynein and Kar9 activity. Namely these assays require some additional controls to properly support the authors' conclusions. The second area of concern are apparent inconsistencies in the She1 in vitro microtubule binding assays that need to be addressed. A third, more general issue is the significant number of assays that were conducted only twice. Although new findings need to be replicated at a minimum of once, this is the absolute minimum and the vast majority of findings are replicated more than twice in published work. While it is not unusual to see confirmatory experiments, or highly resource draining experiments repeated only twice, the relatively large number of experiments replicated only twice dampens the impression and distracts from the otherwise high-quality nature of the data and presentation.

Overall, my comments fall into three categories: Major Comments that this reviewer feels need to be addressed in order to support the conclusions drawn by the authors, Significant Comments which should be addressed as much as reasonably possible to produce a study of sufficient transparency/reproducibility (most of which are likely addressed via text addition or clarification), and Minor Comments.

Lastly, the authors report a very interesting finding that the G437R mutation may potential allosterically disrupt MAP binding on other native tubulin heterodimers within the same microtubule. This is an exciting model and with significant implications for understanding how mutations in a single isotype can alter MT function. Currently, however, this finding is based largely on the interpretation of indirect evidence. While not essential for the publication of this manuscript, the authors may have the tools in hand to directly test this, which would significantly strengthen this insightful conclusion. Thus, the topic is placed in the Significant Comments, rather than Major Comments section.

Major Comments:

The study draws conclusions about the relative function of the Kar9 and Dynein spindle positioning pathways. However, the description of how each contributes to spindle position versus orientation is overly simplified and the function of either pathway is not evaluated in a sufficient nor balanced manner. Essentially, the role of positioning the spindle near the neck is ascribed to Dynein and orienting the spindle to Kar9. In reality, many G2/M spindles are positioned far from the neck in kar9 mutants, as well as well away from the neck in dynein mutant cells (e.g. Hwang et al., 2003, PMID: 12743102; Kusch et al., 2002, PMID: 12101122). Thus, to support the conclusions reached in this study, spindle positioning must not only be compared between wildtype and tub1-G437R cells, but the assays must also include dyn1 mutant and kar9 mutant cells (e.g. in Fig. 4G, Fig S4d). This comment is regarding the evaluation/conclusions of kar9 vs dynein function. The hyperactive dynein-dependent sliding activity in the G437R mutant is well-tested in dynein mutant cells (i.e. Fig 5a-b).

As described for Figs. 4g and S4d above, the analysis of spindle orientation in HU arrested Tub3-only cells (Fig. 8c-d, Fig. S6c) is not sufficient to support the conclusions regarding function of Kar9 vs. Dyn1 spindle positioning. Spindles located far from the neck can result from both dyn1 and kar9 mutations, as well as perturbation of location and orientation near the neck (e.g. Hwang et al., 2003, PMID: 12743102; Kusch et al., 2002, PMID: 12101122). Similarly, an overzealous Kar9 pathway may disrupt spindle location and/or orientation (e.g. Hwang et al., 2003, PMID: 12743102). In order to draw these conclusions that separate the roles of Kar9 and Dyn1 function at this stage of the cell cycle, these results need to be compared to controls performed in cells containing mutations in Dyn1 and Kar9 modulators, and ideally in Tub3-only cells containing the Dyn1 and Kar9 mutations.

One important role of She1 in G2/M is to crosslink and stabilize spindle microtubules (Zhu et al., 2017, PMID: 28794129). Thus, She1 may have increased affinity to bundled or clustered MTs. This is relevant in the in vitro reconstitution assays where it appears that wildtype tubulin polymerizes more readily and produces more MTs than the G437R mutant tubulin. Many of the

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areas with increased She1 binding in Fig. 6d appear to be regions with multiple closely spaced MTs and/or MT crossover. Since the yeast tubulin/MTs are not visible, how is this controlled for? It seems these areas should be excluded, and perhaps only single MTs with a seed attached should be considered. Why was 7 ul She1 sample utilized? How big are flow chambers? Is 7 ul sufficient to completely exchange the chamber, or might there be more binding near the entry end of the chamber? Was this controlled for in any way? Moreover, it is unclear what method was used to quantify She1 binding. It says 60 MTs per type. Is that the average all along MT, or total, or per unit length, etc? Can the She1 values be normalized to tubulin/MT intensity in some way?

A similar issue needs to be considered in Fig 7e. The overall She1 binding to Tub1 vs Tub3 is reported to be similar. Yet the representative images show a difference as great or greater than that apparent between wildtype and G437R in Fig. 6d. How many MTs were scored from each image, and how many images per reaction chamber, from how many trials? It appears these two chambers, or regions of chambers, are quite different. Yet the overall average is similar in 7d and different in 6d. How were the issues outlined above, and the apparent differences in Fig. 7e controlled?

The assessment of viability for Tub1-G437R and tub3 deletion double mutants is based on approximately 6 tetrads. While that showed 6 out of 6 double mutants are inviable, this is an unusually low number on which to base a published result. The minimal standard in the field would typically be ~20 tetrads to draw similar conclusions.

Significant Comments:

The conclusion that Tub1-G437R tubulin allosterically modifies the MT lattice in a manner that prevents MAPS (i.e. She1) from properly interacting with other, native, tubulin subunits is an exciting model and potentially quite impactful for understanding how mutations in specific isotypes alter MT function and cause various disease. Yet, it is based largely on interpretation of indirect evidence, which limits its impact. The authors may have the tools in hand to test this in one of multiple ways, which would significantly strengthen this unexpected, but potential central conclusion. For instance, they may be able to spike microtubules made from purified Tub1 (or Tub3) with increasing amounts of Tub1-G437R. Presumably, a small sub-stoichiometric amount of Tub1-G437R would have a proportionally much larger effect on inhibiting She1 binding to the overall polymer. A similar approach may be possible using the concentrated in vitro lysate assay.

Figure 2: It should be stated clearly whether the dynamicity values are presented as the average of the dynamicity calculated for each MT, or for each cell, or perhaps another method.

The axis on Fig. S3b is not clear. Does this refer to the (1) fraction of cells that contain a MT of indicated length range? Or (2) the fraction of all MTs (of the total population) that are above this range? How were the error bars generated for such a graph? Are they based on individual cells or trials? How many cells or trials were included? Also, are MT number scored as per cell or per SPB, as presumably the G2/M cells will each have 2 SPBs whereas G1 cells have only 1 SPB. This information should be included in the methods or legend.

In Figs. 3a, 3b, one would assume so, but it is important to clarify whether each western blot comes from an independent culture/lysate, and whether the Stain-Free imaging was also quantified or simply used as a visual confirmation of equal loading.

The reduction of tubulin levels stated in the text do not appear to match the graphs shown in Fig. 3b. Specifically, the reduction in Tub1-G437R cells measured with 4A1 antibody appears to be more than 23.5% on the graph. The reduction in the tub3 deletion strain with YL 1/2 antibody does not appear to be 36.9% on the graph in Fig 3b.

The number of MTs per SPB stated in the text do not match the numbers presented in Fig. 3g. Specifically, the number of MTs per spindle pole body is not 34 for mutant and 40.5 for wildtype. Perhaps this is the total number on both SPBs?

In Fig. 4E, the authors should describe how catastrophes were judged to follow a sliding event? Did they need to occur during the sliding? If not, then how long afterward would be considered?

Which cell cycle stages are measured in Fig. 4d and e? The panel in Fig. 4c shows all cell types. Presumably the quantified cells should all be G2/M, but this should be clear in the legend and/or methods. It is important to control for cell cycle stage as Dyn1 localization can differ on MT +ends throughout the cycle (e.g. Grava et al., 2006, PMID: 16580990).

Can the authors report the molar ratio of tub1 c-term peptides used to compete off tubulin from the She1 fragment in the pull downs in Fig. 6C?

In the She1 in vitro MT binding assays, how does the amount of free tubulin (which is more in the mutant, and can bind She1) affect the She1 available for MTs? Following polymerization can this be washed out? Alternatively, perhaps one could flow in G437R mutant cell lysate after She1 is already bound to either WT, tub1 or tub3 only MTs and monitor whether the MT binding is altered by the soluble tubulin type and/or level? The authors should at least discuss this possibility with regard to their data and interpretations.
Minor Comments:

Showing all data points in Fig. 2 is commendable, the experiment appears robust, and the text is very clear. However, the authors should at least report the number of cells analyzed and/or the time analyzed per cell and/or MT number. This information seems to not be listed in the legend, methods or text.

In Fig S3 (a-c) the authors should clearly indicate which stage of the cell cycle the parameters were measured, e.g. G1 or G2-M. (especially since there are differences between G1 and G2-M reported in Fig. 2).

Title of Fig. S4 overstates the conclusion that microtubule number is not altered. The data in this figure shows only GFP-Bim1 localization. The title should be adjusted to accurately reflect what is shown in the figure or additional information supporting the conclusion should be added to the legend.

In Fig. 4 are the cells HU arrested or not? How were they selected?

How were the pHIS3-tubulin constructs expressed? Was the promoter used constitutively or was it specifically induced with -His media before imaging? If so, for how long, and was the level of expression in various strains evaluated somehow?

Page 21, could the authors explain, at least in the methods, how RFP-tubulin is used relative to mRUBY2-tubulin? Is this the starting construct for making pHIS3mRUBY2-TUB1+3'UTR::LEU2, or was it used directly? Is there specific reason to use RFP in some cases and mRUBY in others? It would be helpful to describe how the RFP-TUB1 is used in this study.

This study uses multiple fluorescent proteins (FP) attached to both Tub1 or Tub3. Please confirm whether the linker regions between the FPs and tubulins were identical in all cases or specifically where they were different.

Although this is not critical, the MT isotype incorporation data shown in Fig. 3d would likely be strengthened by including a strain containing an exogenous copy of wild-type mRUBY2-Tub1. Presumably the incorporation of wild-type mRUBY2-Tub1 would also increase in the presence of endogenous Tub1-G437R.

Bottom of page 15: “These findings suggest that the increased spindle intensity values for She1 in TUB3-only cells may largely be a consequence of the increased microtubule number.” It seems straightforward to test this idea by normalizing She1 intensity to MT number or total MT length in the wildtype, G437R, and Tub3-only spindles.

Fig. 3e may be improved if the labels of ‘spindle microtubules’ and ‘astral microtubules’ can be added to clearly demark the two boxes. Currently it may not be straightforward for a reader to follow the boxed and enlarged diagrams, or perhaps have the lines extend from the spindle and astral MTs to the two different MT ratios.

Is there any known reason or possible explanation for why the two antibodies report a stark difference in tubulin levels in the tub3 deletion strain in Fig. 3b? Could it be expected due to differential isotype-specific binding of one or both antibodies, or other possible explanations?

Recognizing that EM tomography of spindles is not trivial, and thus the relatively low sample sizes for spindles in this study, the authors should at least acknowledge in the text that their conclusion that wildtype and Tub1-G437R mutant spindles are largely indistinguishable is based on a very small observed sample.

In Fig S2 it may be helpful to readers to specify which direction the rotation or 90 degrees is made. The arrow may give the impression that the rotation is upward, not to the right.

Page 9, states reduced by 40-61% (in astral and spindle MTs, respectively). This should most likely be stated separately as “40 and 61% in astral and spindle MTs, respectively” if they represent specific values and not a range.

In Fig 4g, the meaning of the bar with p values is unclear. Does this mean that wildtype versus G437R is P<0.0001 for both normal and abnormal categories? Can this be explained in the legend?

Near bottom of page 15 the sentence containing “three of the four models..., while the third possessed” I believe should state ‘while the fourth possessed’.

The tub1D: TEFp::TUB3 and TUB1 TEFp::TUB3 strains are very sick. Although they are used for analysis, it should at least be addressed in the text that observed phenotypes could be to an unknown extent confounded by the sickness of the cell.

Discussion, page 19: It would increase clarity if the sentence read, “overexpression of Tub3 leads to cells with greater numbers of ‘spindle’ microtubules.”

Page 16, states: “Mitotically arrested cells possess greater numbers of microtubules emanating from each SPB. Thus we
wondered whether the increased microtubule number in the TUB3-only cells is a consequence of a mitotic arrest phenotype. To determine whether this is the case, we quantitated mitotic duration by measuring...". This will not directly show whether this is the case. It will rather reveal a delay in duration or not, which may or may not be responsible for the increased MT number. Clarity may also be improved if the authors explain why they are uncertain whether the 26% delay may be responsible. How much time would be predicated to be needed if the delay were responsible? If not due to the delay are there other reasonable and/or known explanations, e.g. could the shorter delay build up SPB size and/or MT number over successive cell cycles? Or does the older SPB no longer expand in subsequent cycles?

Typo in Fig. 8 legend: The rose plot is represented in panel d (not c).

Page 6: Clarifying what is meant by an "allosteric effect" of a MAP on the MT can be useful for new readers. It can be misinterpreted as a local change of one tubulin heterodimer's allosteric conformation and not the long-range global MT effect that the authors propose at the end of the study.

Middle of page 5: Markus and Lee, 2011 cited twice in a row.

Middle of page 5: The sentence "Dynein activity is largely governed by its localization to these sites; however, as in higher eukaryotes, at least one known MAP also regulates dynein activity in cells: She1." appears somewhat ambiguous in that it can give the impression that She1 operates both in yeast and higher eukaryotes. Perhaps it may be helpful to clarify this.

On page 8, The statement should read that a reduction in MT mass would 'likely' lead to... (e.g. otherwise test it in the deletion cells).

Page 5, second paragraph, first sentence: It may be helpful to non-expert readers to specify that this description of Dynein function is regarding pre-anaphase cells/spindle (i.e. during anaphase Dynein will pull the SPB into the bud rather than position at the neck).

Page 21, typo near bottom: “relative alpha-tubulin incorporation”; is written as “relative a-tubulin incorporation”.

Reviewer #2 (Remarks to the Author):

This is a review of the manuscript submitted by Denarier and Ecklund and colleagues, “Modeling a disease-correlated tubulin mutation in budding yeast reveals insight into MAP-mediated dynein function”. The paper has obviously been put together with care - the text is generally well-written and the figures are polished. I will focus on three areas in my review: the rationale, and the two main experimental threads (dynein branch and tubulin isotype usage branch).

The foundational approach of the paper was to express the G437R (yeast G437 is equivalent to the G436 in human a-tubulin) mutant in Tub1, the major a-tubulin in budding yeast. Fig. 1 shows the location of the mutation (the microtubule cartoon is very nice even if it only has 12 protofilaments) and documents that it is tolerated by cells with some hypersensitivity to benomyl, and can be incorporated into microtubules. I thought the use of the phrase ‘polymerization competent’ in the title for Fig. 1 was maybe too strong given later results from the paper. Fig. 2 (which is well-prepared but mighty ‘busy’) shows the results from measuring microtubule dynamics in wild-type and tub1-G437R mutant strains. Mutant microtubules showed G2/M-specific increases in growing and shrinking rates, as well as some other G2/M-specific changes in transition frequencies.

Figs 4 and 5 compare spindle movements between wild-type and tub1-G437R cells, with Fig. 4 documenting a number of changes including much more mobile spindles in mutant cells, and Fig. 5 using double mutant analysis to show that the
enhanced spindle displacement can be ascribed to dynein, via She1. Both of these figures seemed well-done. The authors might consider merging them (relegating some panels to supplemental) to streamline the paper somewhat.

Figures 6 and 7 go after She1 interactions with microtubules, using two-hybrid, pulldown, and in vitro binding-by-microscopy assays (Fig. 6) as well as by quantifying the intensity of GFP-She1 on spindles (Fig. 7). The experiments are well-done and it was nice to see an application of the “microtubules grown from extract” methods developed by the Barnes/Drubin labs. The data support the model in which the impact of the mutation on dynein function occurs because the mutation interferes with interactions of the dynein adaptor protein She1 with microtubules.

Figures 3 and 8 represent a second thrust of the paper that has to do with α-tubulin isotype usage in yeast. This aspect of the manuscript is not as well-developed as the dynamics/She1/dynein part. They found using immunoblotting and fluorescence that the ‘minor’ α-tubulin isotype Tub3 makes up a higher fraction of total α-tubulin (~50%) compared to prior reports (~20%). There was little commentary to explain or speculate about the possible origins of the discrepancy, and the overall treatment seemed somewhat anecdotal here. Given that the paper is already on the long side, it might be a good idea to save this part for some future paper, only keeping what is needed to directly support the She1/dynein story.
We would like to thank both the reviewers for their very thoughtful and thorough review of our manuscript. They clearly have a very strong grasp of the literature and biology, and have put a lot of thought and effort into their review. Their comments and concerns have indeed forced us to make some changes and additions that have significantly strengthened our manuscript. We have addressed their comments in detail below.

Reviewer #1

1) "A third, more general issue is the significant number of assays that were conducted only twice. Although new findings need to be replicated at a minimum of once, this is the absolute minimum and the vast majority of findings are replicated more than twice in published work. While it is not unusual to see confirmatory experiments, or highly resource draining experiments repeated only twice, the relatively large number of experiments replicated only twice dampens the impression and distracts from the otherwise high-quality nature of the data and presentation."

We would like to clarify that due to the limited amount of time remaining in the lab for one of the lead authors, a small number of the experiments were only performed two times. However, the degree of significance for those particular experiments that were required to support our conclusions (e.g., some in Figs 6 and 7) revealed sufficient differences (i.e., P < 0.0001) that our confidence in the findings were high enough that a third replicate was not necessary. In support of the consistency between replicate datasets, t tests revealed large P values for them (e.g., for Figure 6E, \( P = 0.5943 \) for replicates 1 and 2 for \( TUB1 \), and \( P = 0.8201 \) for replicates 1 and 2 for \( tub1^{G437R} \), compare to \( P < 0.0001 \) for \( TUB1 \) versus \( tub1^{G437R} \)). The same is true for datasets in Figure 7.

Major Comments:

2) "The study draws conclusions about the relative function of the Kar9 and Dynein spindle positioning pathways. However, the description of how each contributes to spindle position versus orientation is overly simplified and the function of either pathway is not evaluated in a sufficient nor balanced manner. Essentially, the role of positioning the spindle near the neck is ascribed to Dynein and orienting the spindle to Kar9. In reality, many G2/M spindles are positioned far from the neck in kar9 mutants, as well as well away from the neck in dynein mutant cells (e.g. Hwang et al., 2003, PMID: 12743102; Kusch et al., 2002, PMID: 12101122). Thus, to support the conclusions reached in this study, spindle positioning must not only be compared between wildtype and tub1-G437R cells, but the assays must also include dyn1 mutant and kar9 mutant cells (e.g. in Fig. 4G, Fig S4d). This comment is regarding the evaluation/conclusions of kar9 vs dynein function. The hyperactive dynein-dependent sliding activity in the G437R mutant is well-tested in dynein mutant cells (i.e. Fig 5a-b)."

We agree with the reviewer that including kar9\( \Delta \) and dyn1\( \Delta \) mutants in our analysis would help to strengthen and/or validate our conclusions. Our revised manuscript now includes analyses of spindle orientation (Figure S4) and position (Figure 4G) from dyn1\( \Delta \) TUB1, dyn1\( \Delta \) tub1\(^{G437R}\), kar9\( \Delta \) TUB1, and kar9\( \Delta \) tub1\(^{G437R}\) cells. Of note, we have found that deletion of either DYN1 or KAR9 alone is sufficient to cause spindle orientation defects, suggesting that spindle orientation is a poor indicator of which pathway a mutation may be affecting. However, in contrast to DYN1 deletion, which causes a large increase in apparent spindle position defects (\( P < 0.0001 \)), deletion of KAR9 alone leads to only a small increase in apparent spindle position defects (\( P = 0.1545 \); see new Figure 4G). Even more importantly, dyn1\( \Delta \) tub1\(^{G437R}\) cells exhibit no more mispositioned spindles than dyn1\( \Delta \) TUB1 cells, whereas kar9\( \Delta \) tub1\(^{G437R}\) cells exhibit significantly more mispositioned spindles than kar9\( \Delta \) TUB1 cells. These data thus strengthen our conclusions, and indicate that tub1\(^{G437R}\) indeed leads to defects in dynein pathway, and not Kar9 pathway function. We thank the reviewer for the suggestion.

3) "As described for Figs. 4g and S4d above, the analysis of spindle orientation in HU arrested Tub3-only cells (Fig. 8c-d, Fig. S6c) is not sufficient to support the conclusions regarding function of Kar9 vs. Dyn1 spindle positioning. Spindles located far from the neck can result from both dyn1 and kar9 mutations, as well as perturbation of location and orientation near the neck (e.g. Hwang et al., 2003, PMID: 12743102; Kusch et al., 2002, PMID: 12101122). Similarly, an overzealous Kar9 pathway may disrupt spindle location and/or orientation (e.g. Hwang et al., 2003, PMID: 12743102). In order to draw these conclusions that separate the roles of Kar9 and Dyn1 function at this stage of the cell cycle, these results need to be compared to controls performed in cells containing mutations in Dyn1 and Kar9 modulators, and ideally in Tub3-only cells containing the Dyn1 and Kar9 mutations."

As noted above, we have now included assessments of spindle orientation and position in dynein and Kar9 pathway mutants in our revised manuscript (see point 2 above). Given our observations described above – that spindle position is a fairly good read-out of dynein pathway dysfunction, but spindle orientation is not – our findings with TUB3-only cells (that spindle orientation, but not position is affected) indeed support the notion that Kar9 and not
dynein function is likely disrupted in TUB3-only cells. To gain additional insight into which pathway may be disrupted, our revised manuscript now includes new data in which the health of TUB3-only cells with either KAR9 or DYN1 deletions is determined. These new synthetic genetic interaction data reveal that combination of either kar9Δ or dyn1Δ with the TUB3-only genotype leads to cells with somewhat compromised viability. However, the health of TUB3-only dyn1Δ cells is more compromised than TUB3-only kar9Δ cells (Figure 8E), suggesting that Kar9 pathway function is the more likely target of disruption in TUB3-only cells. Given the indirect nature of these assays, we have softened the language describing these conclusions.

4a) “One important role of She1 in G2/M is to crosslink and stabilize spindle microtubules (Zhu et al., 2017, PMID: 28794129). Thus, She1 may have increased affinity to bundled or clustered MTs. This is relevant in the in vitro reconstitution assays where it appears that wildtype tubulin polymerizes more readily and produces more MTs than the G437R mutant tubulin.”

In contrast to the image shown in our original Figure 6D, wild-type tubulin does not polymerize more readily than the mutant tubulin. The number of microtubules per field are in fact directly proportional to the number of microtubule seeds that are adhered to the coverglass. For each replicate, we used the same microtubule seed mixture from which to nucleate control and mutant microtubules, and one of these replicates had a lower apparent seed concentration than the other. Unfortunately, our original draft showed one image each from distinct replicates (wild-type from the higher seed concentration replicate, the mutant from the lower seed concentration). We have replaced the images with those that are more reflective of images used for direct comparison (from only one of the replicates). We apologize for selecting images that were indeed misleading, and we thank the reviewer for pointing this out.

That being said, we were careful to assess She1-microtubule binding on those microtubules that were not bundled (see points 4b and c below).

4b) “Many of the areas with increased She1 binding in Fig. 6d appear to be regions with multiple closely spaced MTs and/or MT crossover. Since the yeast tubulin/MTs are not visible, how is this controlled for? It seems these areas should be excluded, and perhaps only single MTs with a seed attached should be considered.”

To clarify, the microtubules generated by this technique (from concentrated cell extracts and GMPCPP-stabilized seeds) were indeed visible by interference reflection microscopy (IRM; see Figure SSD for example). For data presented in Figures 6E and 7F, we focused our fluorescence She1 intensity measurements on regions of microtubules that excluded overlaps, intersections, and bundles. Please see our answer to point 4c below for more information.

4c) "Why was 7 ul She1 sample utilized? How big are flow chambers? Is 7 ul sufficient to completely exchange the chamber, or might there be more binding near the entry end of the chamber? Was this controlled for in any way? Moreover, it is unclear what method was used to quantify She1 binding. It says 60 MTs per type. Is that the average all along MT, or total, or per unit length, etc? Can the She1 values be normalized to tubulin/MT intensity in some way?"

To clarify, we used only 1 µl of purified She1 for the in vitro microtubule-binding assays described by the reviewer. To address the reviewer's other questions, we have added additional information (denoted in highlighted text) to the Methods section as follows: “To assess She1-microtubule binding, 1 µl of full-length She1-HaloTag260 diluted in 7 µl of clarified lysate (supplemented with 0.5 mM GTP) was then introduced into the imaging chamber with the polymerized microtubules [note that each chamber accommodates approximately 7-8 µl in volume; thus, ~1 chamber volume of She1-supplemented lysate was introduced into each chamber]. Lysate was used instead of buffer to ensure that sufficient tubulin concentrations were present to prevent coverslip-immobilized microtubules from depolymerizing. Images of She1-HaloTag260, microtubules (acquired using interference fluorescence microscopy, or IRM; see Fig SSD for example), and rhodamine seeds were acquired using TIRF microscopy immediately following addition into chamber (all images were acquired within 3 minutes of adding She1). Using ImageJ, lines drawn along the length of single, non-bundled microtubules (identified in the IRM channel) were used to determine the mean fluorescence intensity per pixel. Overlapping and/or intersecting microtubules were omitted from our measurements, as were microtubule seed regions, to which we noted She1 binds to a significantly lower extent (e.g., see arrowheads in Figures 6D or 7E). Approximately 4-6 different fields were acquired to control for potential variation between fields of view. To control for potential regional She1 concentration differences within each chamber, images were generally acquired within the middle of each, a significant distance from the sample loading area. Note that replicates were performed on different days.”

Given the consistent differences between wild-type and mutant tubulin extracts across our two replicates, we are confident in our results.
Unfortunately, our methodology does not permit us to normalize She1 intensity values to microtubule intensity values due to limitations with IRM, which is not necessarily quantitative or even across a field of view.

5) "A similar issue needs to be considered in Fig 7e. The overall She1 binding to Tub1 vs Tub3 is reported to be similar. Yet the representative images show a difference as great or greater than that apparent between wildtype and G437R in Fig. 6d. How many MTs were scored from each image, and how many images per reaction chamber, from how many trials? It appears these two chambers, or regions of chambers, are quite different. Yet the overall average is similar in 7d and different in 6d. How were the issues outlined above, and the apparent differences in Fig. 7e controlled?"

We apologize for including an image in Figure 7E that is not necessarily representative of the mean of the data set. As stated in Figure 7, we measured She1 intensity along 60 microtubules for each condition (from 4-6 images; note we did not observe significant deviations from image-to-image), from two independent replicates (using the methods described above in point 4c). As shown in the scatter plot overlay in Figure 7F, there is indeed a range of intensity values for each data set (as is normally the case for such an experiment). Images were acquired, and measurements were performed using carefully controlled, and identical methods for each condition. We have replaced the images in Figure 7E with some that are more representative of the mean of each. We apologize for this point of confusion, and thank the reviewer for pointing this out.

6) "The assessment of viability for Tub1-G437R and tub3 deletion double mutants is based on approximately 6 tetrads. While that showed 6 out of 6 double mutants are inviable, this is an unusually low number on which to base a published result. The minimal standard in the field would typically be ~20 tetrads to draw similar conclusions."

To clarify, we obtained 6 inviable double mutants from 12 separate tetrads. We apologize for the confusion. However, we have now dissected an additional 12 tetrads (24 total), from which 6 additional spores were inviable that were expected to be double mutants. Given that we have now obtained 12 out of 12 inviable spores, we are confident in these findings. Our manuscript has been updated to reflect this.

Significant Comments:

7) "The conclusion that Tub1-G437R tubulin allosterically modifies the MT lattice in a manner that prevents MAPS (i.e. She1) from properly interacting with other, native, tubulin subunits is an exciting model and potentially quite impactful for understanding how mutations in specific isoforms alter MT function and cause various disease. Yet, it is based largely on interpretation of indirect evidence, which limits its impact. The authors may have the tools in hand to test this in one of multiple ways, which would significantly strengthen this unexpected, but potential central conclusion. For instance, they may be able to spike microtubules made from purified Tub1 (or Tub3) with increasing amounts of Tub1-G437R. Presumably, a small sub-stoichiometric amount of Tub1-G437R would have a proportionally much larger effect on inhibiting She1 binding to the overall polymer. A similar approach may be possible using the concentrated in vitro lysate assay."

We agree this would be interesting experiment that would strengthen our conclusion regarding the potential allostry imparted on the microtubule lattice by the tubulin mutant. However, given limitations with respect to the lead author graduating, and the effort that such an experiment would take to perform properly, we think these experiments are beyond the scope of this current manuscript.

8) "Figure 2: It should be stated clearly whether the dynamicity values are presented as the average of the dynamicity calculated for each MT, or for each cell, or perhaps another method."

We have clarified this point in the revised manuscript as follows (revisions denoted in highlighted text): “For each cell a single microtubule was used for dynamics measurements (n = 41 and 42 microtubules – from the same number of cells – from G1 TUB1 and tub1G437R cells, respectively, and n = 31 and 53 microtubules/cells from G2/M TUB1 and tub1G437R, respectively).” We have also edited the legend of Figure S3B to include the following point of clarification: “Note that microtubule length measurements were performed from the first time point of movies used for microtubule dynamics assessments (see Figure 2).”

9) "The axis on Fig. S3b is not clear. Does this refer to the (1) fraction of cells that contain a MT of indicated length range? Or (2) the fraction of all MTs (of the total population) that are above this range? How were the error bars generated for such a graph? Are they based on individual cells or trials? How many cells or trials were included? Also, are MT number scored as per cell or per SPB, as presumably the G2/M cells will each have 2 SPBs whereas..."
G1 cells have only 1 SPB. This information should be included in the methods or legend.

The plot in S3B shows the aggregate fractions of all microtubules from G2/M cells from three biological replicates with the indicated length range. The error bars represent the standard error of proportion, as is common for such datasets. We apologize for omitting these pieces of information, which has now been included in the legend for Figure S3.

10) "In Figs. 3a, 3b, one would assume so, but it is important to clarify whether each western blot comes from an independent culture/lysate, and whether the Stain-Free imaging was also quantified or simply used as a visual confirmation of equal loading."

We apologize for omitting this piece of information. The replicates are from independent lysates prepared from independent cell cultures. We have modified the figure legend to reflect this. Moreover, the stain-free imaging was used to determine the degree of loading. This latter point is noted in the Methods section: "Bio-Rad Stain-Free gel signal was used to correct for differences in lysate loading between lanes."

11) "The reduction of tubulin levels stated in the text do not appear to match the graphs shown in Fig. 3b. Specifically, the reduction in Tub1-G437R cells measured with 4A1 antibody appears to be more than 23.5% on the graph. The reduction in the tub3 deletion strain with YL1/2 antibody does not appear to be 36.9% on the graph in Fig 3b."

We apologize for our mistake. We have revised the text to include the correct values: "30.55 - 42.7% reduction, determined with 4A1 and YL1/2, respectively."

12) "The number of MTs per SPB stated in the text do not match the numbers presented in Fig. 3g. Specifically, the number of MTs per spindle pole body is not 34 for mutant and 40.5 for wildtype. Perhaps this is the total number on both SPBs?"

We apologize for our mistake. We indeed meant microtubules per spindle, not per SPB. The text has been revised accordingly.

13) "In Fig. 4E, the authors should describe how catastrophes were judged to follow a sliding event? Did they need to occur during the sliding? If not, then how long afterward would be considered?"

We apologize for the lack of details regarding this analysis. We have added the following information to the Materials and Methods: "Catastrophe events were defined as “following a sliding event” (Figure 4E) if they occurred during the 1 minute period following the cessation of a sliding event." We thank the reviewer for pointing out this omission on our part.

14) "Which cell cycle stages are measured in Fig. 4d and e? The panel in Fig. 4c shows all cell types. Presumably the quantified cells should all be G2/M, but this should be clear in the legend and/or methods. It is important to control for cell cycle stage as Dyn1 localization can differ on MT +ends throughout the cycle (e.g. Grava et al., 2006, PMID: 16580990)."

We apologize for omitting this important piece of information. We have added the following information to the legend of Figure 4: "For all panels, G2/M cells (determined based on morphological criteria, i.e. those with a bud diameter ≥ 75% of the mother cell) were selected for analysis." We thank the reviewer for pointing this out.

15) "Can the authors report the molar ratio of tub1 c-term peptides used to compete off tubulin from the She1 fragment in the pull downs in Fig. 6C?"

We apologize for omitting this piece of information. We have included this information in the revised Materials and Methods (i.e., we used an approximate 900-molar excess of peptide with respect to She1C-term).

16) "In the She1 in vitro MT binding assays, how does the amount of free tubulin (which is more in the mutant, and can bind She1) affect the She1 available for MTs? Following polymerization can this be washed out? Alternatively, perhaps one could flow in G437R mutant cell lysate after She1 is already bound to either WT, tub1 or tub3 only MTs and monitor whether the MT binding is altered by the soluble tubulin type and/or level? The authors should at least discuss this possibility with regard to their data and interpretations."
We indeed attempted to include a microtubule-stabilizing agent into the chambers so that we could wash out the free tubulin prior to addition of She1. However, we encountered technical difficulties doing so, and instead used the strategy described in the methods (purified She1 was diluted into concentrated cell lysate, which was then added to the chamber as described in detail in our revised manuscript, and in point 4c above). Thus, we are unable to account for a potential reduction in She1-microtubule binding as a consequence of She1-tubulin binding in these experiments.

Although we appreciate the reviewer’s suggestion (flowing in tub1<sup>G437R</sup> mutant cell lysate after She1 is already bound to microtubules), we worry this may complicate interpretation of the microtubule-binding assays given the mutant tubulin will indeed be added to the ends of existing microtubules, or potentially even be incorporated into the lattice of pre-existing microtubules (as has been noted in the recent literature).

Minor Comments:

17) “Showing all data points in Fig. 2 is commendable, the experiment appears robust, and the text is very clear. However, the authors should at least report the number of cells analyzed and/or the time analyzed per cell and/or MT number. This information seems to not be listed in the legend, methods or text.”

As described above in point 8, we have included this information in the revised manuscript.

18) “In Fig S3 (a-c) the authors should clearly indicate which stage of the cell cycle the parameters were measured, e.g. G1 or G2-M. (especially since there are differences between G1 and G2-M reported in Fig. 2).”

As described above in point 9 (and in the revised figure legend), only G2/M cells were used for this analysis.

19) “Title of Fig. S4 overstates the conclusion that microtubule number is not altered. The data in this figure shows only GFP-Bim1 localization. The title should be adjusted to accurately reflect what is shown in the figure or additional information supporting the conclusion should be added to the legend.”

We have revised the text as suggested. We thank the reviewer for the suggestion.

20) “In Fig. 4 are the cells HU arrested or not? How were they selected?”

The cells used for analysis in in Figure 4 were not HU-arrested. As stated above in point 14, only G2/M cells were used for these analyses.

21) “How were the pHIS3-tubulin constructs expressed? Was the promoter used constitutively or was it specifically induced with -His media before imaging? If so, for how long, and was the level of expression in various strains evaluated somehow?”

We apologize for not including this detail. Our revised manuscript now includes the following note: “Prior to imaging cells with these chromosomally-integrated FP-Tub1 plasmids, cells were grown in synthetic defined complete media (i.e., histidine was indeed included, omission of which would potentially lead to increased levels of expression from the HIS3 promoter (Kim and Clark, 2002)).” We thank the reviewer for pointing out this omission.

22) “Page 21, could the authors explain, at least in the methods, how RFP-tubulin is used relative to mRUBY2-tubulin? Is this the starting construct for making pHIS3:mRUBY2-TUB1+3’UTR::LEU2, or was it used directly? Is there specific reason to use RFP in some cases and mRUBY in others? It would be helpful to describe how the RFP-TUB1 is used in this study.”

The reason for the two plasmids being used is simply a consequence of the collaboration. The Boscheron lab employed the pAFS125-derived RFP-Tub1 plasmid for their experiments (those shown in Figures 1C, 5C – E), while the Markus lab employed pHIS3p:mRuby2-tub1G437R+3’UTR::LEU2 (employed in Figures 3C and D). This information has been added to the revised methods section. We thank the reviewer for raising this point.

23) “This study uses multiple fluorescent proteins (FP) attached to both Tub1 or Tub3. Please confirm whether the linker regions between the FPs and tubulins were identical in all cases or specifically where they were different.”

For the quantitative imaging experiments comparing mRuby2-Tub1 to mRuby2-Tub3 (Figure 3C and D), the plasmids indeed encoded identical linker regions between the mRuby2 and the Tub1/Tub3. We have modified the
text as such: “note that both mRuby2-TUB1 and mRuby2-TUB3 possess a glycine-serine linker between the mRuby2 and the tubulin gene.”

24) “Although this is not critical, the MT isotype incorporation data shown in Fig. 3d would likely be strengthened by including a strain containing an exogenous copy of wild-type mRUBY2-Tub1. Presumably the incorporation of wild-type mRUBY2-Tub1 would also increase in the presence of endogenous Tub1-G437R.”

We agree that this would provide additional evidence that the mutant Tub1G47R is incorporated to a lesser extent than a wild-type α-tubulin (Tub1 in this case). Although we appreciate the great suggestion, in light of the evidence we have already obtained, we don’t think this experiment will substantially strengthen our conclusions.

25) “Bottom of page 15: "These findings suggest that the increased spindle intensity values for She1 in TUB3-only cells may largely be a consequence of the increased microtubule number." It seems straightforward to test this idea by normalizing She1 intensity to MT number or total MT length in the wildtype, G437R, and Tub3-only spindles.”

We indeed attempted to normalize GFP-She1 spindle intensity to mRuby2-Tub1 (or Tub1G437R) intensity values. It was at this point that we started noting differences in spindle intensities between the wild-type and mutant tubulin (e.g., due to lower incorporation of Tub1G437R into cellular microtubules), which led to complications in the normalization procedure (and was the basis for our further investigating these values with respect to each other). Moreover, for reasons that are currently unclear, we are unable to visualize microtubules in TUB3-only cells with mRuby2-Tub3, thus rendering this normalization impossible. That being said, we appreciate the great suggestion.

26) “Fig. 3e may be improved if the labels of ‘spindle microtubules’ and ‘astral microtubules’ can be added to clearly demark the two boxes. Currently it may not be straightforward for a reader to follow the boxed and enlarged diagrams, or perhaps have the lines extend from the spindle and astral MTs to the two different MT ratios.”

We apologize for the confusion, but Figure 3E depicts the ratio of Tub1:Tub3 in all cellular microtubules. Our values depict a range of values (e.g., 42-46% Tub1:Tub2: 54-58% Tub3:Tub2) in wild-type (TUB1, top) or mutant (tub1G437R, bottom) cells. We do not distinguish astral from spindle microtubules in this drawing. In an attempt to clarify this point, we have modified Figure 3E by indicating “cellular microtubules” above the drawing. We hope this helps.

27) “Is there any known reason or possible explanation for why the two antibodies report a stark difference in tubulin levels in the tub3 deletion strain in Fig. 3b? Could it be expected due to differential isotype-specific binding of one or both antibodies, or other possible explanations?“

The reason for the differences in our immunoblotting results noted with the two antibodies is unclear. Although purely conjecture, our hypothesis is the same as the one posited by the reviewer: that the antibodies exhibit different affinities for Tub1 and Tub3. We hope to one day have an answer to this question.

28) “Recognizing that EM tomography of spindles is not trivial, and thus the relatively low sample sizes for spindles in this study, the authors should at least acknowledge in the text that their conclusion that wildtype and Tub1-G437R mutant spindles are largely indistinguishable is based on a very small observed sample.”

We have revised the text to include this caveat.

29) “In Fig S2 it may be helpful to readers to specify which direction the rotation or 90 degrees is made. The arrow may give the impression that the rotation is upward, not to the right.”

We have corrected the figure to more accurately reflect the direction and angle of rotation. We hope this helps to clarify things.

30) “Page 9, states reduced by 40-61% (in astral and spindle MTs, respectively). This should most likely be stated separately as “40 and 61% in astral and spindle MTs, respectively” if they represent specific values and not a range.”

We have revised the text as suggested. We thank the reviewer for the suggestion.
31) "In Fig 4g, the meaning of the bar with p values is unclear. Does this mean that wildtype versus G437R is P<0.0001 for both normal and abnormal categories? Can this be explained in the legend?"

We have revised the figure legend as suggested. We hope it is more clear now.

32) "Near bottom of page 15 the sentence containing "three of the four models... while the third possessed" I believe should state ‘while the fourth possessed’.

We have revised the text to correct this mistake. We thank the reviewer for pointing out this mistake.

33) "The tub1D: TEFp::TUB3 and TUB1 TEFp::TUB3 strains are very sick. Although they are used for analysis, it should at least be addressed in the text that observed phenotypes could be to an unknown extent confounded by the sickness of the cell."

Although the sickness and growth defects of the TUB3-only cells are indeed a consequence of the genotype (in particular, the loss of Tub1), our phenotypic analysis is restricted to microtubule-dependent phenotypes (spindle morphology and position/orientation). Any alterations in microtubule-dependent phenotypes are most likely a direct consequence of the alterations to the tubulin genotype. Although other aspects of the cell biology of TUB3-only cells are likely impacted as an indirect consequence of these microtubule-dependent phenotypes (e.g., metabolism, gene expression, etc.), our analysis is focused on those phenotypes most likely directly correlated with the genotype. As a side note, wouldn’t the same be true for all such genetic perturbations? It should be assumed that disruption of any gene may potentially lead to indirect effects, and thus interpretation of results should be done carefully.

34) "Discussion, page 19: It would increase clarity if the sentence read, "overexpression of Tub3 leads to cells with greater numbers of 'spindle' microtubules."

We have revised the text to clarify this point. We thank the reviewer for the suggestion.

35) "Page 16, states: "Mitotically arrested cells possess greater numbers of microtubules emanating from each SPB. Thus we wondered whether the increased microtubule number in the TUB3-only cells is a consequence of a mitotic arrest phenotype. To determine whether this is the case, we quantitated mitotic duration by measuring...". This will not directly show whether this is the case. It will rather reveal a delay in duration or not, which may or may not be responsible for the increased MT number. Clarity may also be improved if the authors explain why they are uncertain whether the 26% delay may be responsible. How much time would be predicated to be needed if the delay were responsible? If not due to the delay are there other reasonable and/or known explanations, e.g. could the shorter delay build up SPB size and/or MT number over successive cell cycles? Or does the older SPB no longer expand in subsequent cycles?"

We agree that correlation is not necessarily indicative of causation. Thus, we have softened the language to read: "To determine whether this may be the case." We have also clarified our thoughts on why 26% may not be sufficient to account for the arrest as follows: "However, compared with the extensive arrest employed with the cdc20-1 mutant cells described above (grown for 4 hours at the restrictive temperature), it is unclear if the short delay (39.9 versus 50.3 minutes) is the basis for the expanded SPB and enhanced microtubule number in these cells."

Although we find the hypotheses posited by the reviewer (i.e., that the short mitotic delay may lead to ‘build up’ of the SPBs over successive cell cycles) to be very reasonable indeed, we don’t have sufficient data to support this idea.

36) "Typo in Fig. 8 legend: The rose plot is represented in panel d (not c)."

We have corrected our mistake. We thank the reviewer for pointing out this mistake.

37) "Page 6: Clarifying what is meant by an "allosteric effect" of a MAP on the MT can be useful for new readers. It can be misinterpreted as a local change of one tubulin heterodimer's allosteric conformation and not the long-range global MT effect that the authors propose at the end of the study."

We have revised the sentence as suggested to read as follows: "Our data suggest that the presence of low copies of the mutant Tub1 α-tubulin protein in microtubules exerts allosteric, long-range effects on microtubule structure that lead to cooperative disruption of She1-microtubule binding, yet protect cells against the severe morphological defects that arise in cells expressing only the compensatory Tub3 α-tubulin isotype."
38) "Middle of page 5: Markus and Lee, 2011 cited twice in a row."

We have corrected our mistake. We thank the reviewer for pointing out this mistake.

39) "Middle of page 5: The sentence "Dynein activity is largely governed by its localization to these sites; however, as in higher eukaryotes, at least one known MAP also regulates dynein activity in cells: She1." appears somewhat ambiguous in that it can give the impression that She1 operates both in yeast and higher eukaryotes. Perhaps it may be helpful to clarify this."

We have revised the sentence to read as follows: "Dynein activity is largely governed by its localization to these sites; however, at least one known MAP also regulates dynein activity in yeast: She1." We thank the reviewer for the suggestion.

40) "On page 8, The statement should read that a reduction in MT mass would 'likely' lead to... (e.g. otherwise test it in the deletion cells)."

We have revised the text as suggested. We thank the reviewer for the suggestion.

41) "Page 5, second paragraph, first sentence: It may be helpful to non-expert readers to specify that this description of Dynein function is regarding pre-anaphase cells/spindle (i.e. during anaphase Dynein will pull the SPB into the bud rather than position at the neck)."

We have revised the text as suggested to read: "...which results in the positioning of the pre-anaphase spindle at the mother-bud neck." We thank the reviewer for the suggestion.

42) "Page 21, typo near bottom: "relative alpha-tubulin incorporation"; is written as "relative a-tubulin incorporation"."

We have corrected our mistake. We thank the reviewer for pointing out this mistake.
Reviewer #2

1) “The lack of more obvious disease relevance is not a fatal flaw - that is how science goes sometimes, and besides the authors made some interesting findings - but there is something of a dissonance between the introduction and the main results, and the discussion seemed overly speculative in its hopeful reaching that a similar mechanism might operate through a She1 functional equivalent. It would be nice if the authors could smooth this aspect of the manuscript out - to be clear I don't think 'disease relevance' has to be purged from the manuscript, but I do think it is a little too prominent in the present text and that this has the effect of undermining the work presented.”

We appreciate the reviewers' perspective; however, we do feel that the discussion is an opportunity to speculate about such things. We would prefer to leave the discussion in question as is.

2) “I thought the use of the phrase 'polymerization competent' in the title for Fig. 1 was maybe too strong given later results from the paper.”

We agree that our results indicate that the mutant tubulin is not capable to polymerizing in the absence of wild-type tubulin. However, given the mutant tubulin is incorporated into microtubules, this indeed indicates that it is polymerization competent.

3) “Figs 4 and 5 compare spindle movements between wild-type and tub1-G437R cells, with Fig. 4 documenting a number of changes including much more mobile spindles in mutant cells, and Fig. 5 using double mutant analysis to show that the enhanced spindle displacement can be ascribed to dynein, via She1. Both of these figures seemed well-done. The authors might consider merging them (relegating some panels to supplemental) to streamline the paper somewhat.”

We appreciate the reviewer’s suggestion to merge Figures 4 and 5. However, for the sake of the narrative of our manuscript, and to avoid overloading a single figure, we have decided to keep them as separate figures.

4) “They found using immunoblotting and fluorescence that the ‘minor’ a-tubulin isotype Tub3 makes up a higher fraction of total a-tubulin (~50%) compared to prior reports (~20%). There was little commentary to explain or speculate about the possible origins of the discrepancy, and the overall treatment seemed somewhat anecdotal here. Given that the paper is already on the long side, it might be a good idea to save this part for some future paper, only keeping what is needed to directly support the She1/dynein story.”

Much like the reviewer, we are mystified by the discrepancy between our data and previous data regarding Tub3 usage. Unfortunately, we have no current guesses as to what the basis might be (although we are certainly open to suggestions!).

Due to the importance of understanding the Tub1/Tub3 isotype usage in wild-type and mutant cells to our understanding of how the tubulin mutant affects the cell biology, we think it is important to keep these data in our manuscript. For example, it would be very difficult, if not impossible, to make some of our conclusions without understanding how Tub3 may or may not contribute to the mutant phenotypes. For instance, without knowing the fraction of microtubules comprised of Tub3 (in either TUB1 or tub1\textsuperscript{G437R} cells), we lack context to understand how She1-microtubule binding is affected either in cells or \textit{in vitro}. Thanks to our analyses, we now know that She1-microtubule binding is not reduced as a consequence of increased incorporation of Tub3 into microtubules in mutant cells. Moreover, data obtained from the TUB3-only cells revealed that dynein function is likely not compromised due to increased incorporation of Tub3 into microtubules in the tub1\textsuperscript{G437R} cells. Finally, thanks to these data, we were also able to conclude that incorporation of low levels of the mutant tubulin may exert long-range allosteric effects on microtubules (see Discussion).
Dear Dr. Markus:

I am pleased to inform you that your manuscript has been accepted for publication in Molecular Biology of the Cell.

The revision has appropriately addressed the concerns of the reviewers and improved the manuscript. The study shows that human disease-correlated mutations in yeast tubulin lead to changes of microtubule properties and to spindle positioning defects in budding yeast, stimulating a discussion about the mechanism by which such mutations may cause human disease.

Sincerely,

Thomas Surrey
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Markus:

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.

Within approximately four weeks you will receive a PDF page proof of your article.

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