Tubulin isotype regulation maintains asymmetric requirement for α-tubulin over β-tubulin

Linnea Wethekam and Jeffrey Moore

Corresponding Author(s): Jeffrey Moore, University of Colorado Anschutz Medical Campus

Review Timeline:

Submission Date:	2022-02-17
Editorial Decision:	2022-04-14
Revision Received:	2022-07-19
Editorial Decision:	2022-08-31
Revision Received:	2022-09-28
Editorial Decision:	2022-10-03
Revision Received:	2022-10-17

Monitoring Editor: Melissa Gardner

Scientific Editor: Dan Simon

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.)

DOI: https://doi.org/10.1083/jcb.202202102

April 14, 2022

Re: JCB manuscript #202202102

Dr. Jeffrey Moore
University of Colorado Anschutz Medical Campus
12801 E 17th Ave
MS8108
Aurora, Colorado 80045

Dear Dr. Moore,

Thank you for submitting your manuscript entitled "Asymmetric requirement for α-tubulin over β-tubulin." Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers agree that your study addresses an important question but differ in their assessment of the degree of advance. We believe this work is appropriate for JCB but also that it requires additional experiments to clarify the nature of the tubulin-containing assemblies and determine if they contain chaperonin and/or folding cofactors. Reviewer #3 asks for more evidence to support the claim that increased α-tubulin, but not β-tubulin, expression leads to equilibration of tubulin levels. This reviewer also raises several important concerns regarding the cell lysis method, calculations of tubulin molecule numbers, and questions whether cell division affects tubulin levels. These and the comments of the other reviewers should be addressed in full as well.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

Figures: Your manuscript may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers

during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Melissa Gardner, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Wethekam and Moore, Asymmetric requirement for α-tubulin over β-tubulin.

A tubulin subunit is a heterodimer of αβ-tubulin. However, there are 2 alpha-tubulin genes but only 1 beta-tubulin gene in the budding yeast. The authors set out to determine the functional significance of this asymmetry in the context of microtubule dynamics and spindle assembly. They genetically alter the expression level of the different tubulin genes, then monitor consequences of microtubules and spindles. The results suggest that budding yeast is more sensitive to the level of β-tubulin for proper function.

Gene isotypes and their differential functions is and historically has been an important question. In this context, the tubulin genes of budding yeast serve as an excellent model to address this question. This reviewer finds that the authors have performed the numerous experiments with rigor, and their writing clear and thoughtful. Nevertheless, the findings and conclusions of this paper do not advance our current understanding of tubulin isotypes and their function.

It has been previously and well-established that budding yeast TUB1 (alpha) and TUB2 (beta) is essential, and Tub3 (alpha) in non-essential. The current work, while introducing the term "tubulin assemblies" to describe aggregates of non-functional tubulins, and establishing concentrations of alpha and beta tubulins in budding yeast, does not shed further light on known observations.

I do not recommend publication in the JCB.

Minor comments:

Fig 1D: Some mutant colonies grow better than wild-type, why?
Fig 1G: How are "coefficient of variation" measure?
Fig 2A: A few time-lapsed images would suffice to show growth or shrinkage.
Fig 2G and 2H: For some measurements, half of the population show the opposite trend. In this context of opposite results in the same measurement, can one make a clear conclusion?
Fig 3E and 4D: Can one define non-functional tubulin aggregates as "tubulin assemblies" to probe further?

Reviewer #2 (Comments to the Authors (Required)):

This manuscript uses a combination of molecular genetics, quantitative light microscopy, and quantitative western blots to

address the question of how the levels of alpha and beta tubulin are (separately) regulated in yeast. The authors conclude that alpha tubulin levels are regulated and kept at levels higher than beta tubulin, in part by having multiple alpha tubulin genes. As part of this work, they show that an excess of beta tubulin is highly detrimental, but excess alpha tubulin has little effect, a conclusion that they reach through a series of complementary experiments.

Overall, this is solid paper: carefully designed, conducted, interpreted, and presented. It uses appropriate approaches to provide a mechanistic answer to an important biological problem. This Discussion is interesting and does a good job of placing this work in the context of other work. I have only two concerns. One is that there are some minor writing issues, which I discuss below. My more significant hesitation is that the mechanism that the authors identify seems likely to be specific to yeast, or at least not shared with mammals, as the authors themselves discuss in the Discussion. I personally don't think that this is a problem because many aspects of cell biology that have seemed different in yeast have turned out to provide insight into mammalian and other systems (the actin/myosin-based nature of membrane transport in yeast is one example). In my opinion, the Discussion addresses this issue sufficiently (I would have been much more concerned if the Discussion had not addressed this issue head-on).

Writing:
1. The quality of writing in the Abstract and Introduction is not quite up to that in the rest of the paper. Here are a few examples from the first two pages (this list is not exhaustive)
a. The order in which ideas are presented in the Abstract is confusing. For example, the first description of an observation states, "We find that α-tubulin gene copy number is important for maintaining an excess α-tubulin protein compared to β-tubulin protein and preventing accumulation of super-stoichiometric β-tubulin." It seems to this reader that this statement (and others following) would make more sense if the authors first stated that they observe that α-tubulin is maintained in an excess. Regardless of how the authors edit the abstract, I do think that it needs some work.
b. The abstract should include the fact that the observations were made in S. cerevisiae.
c. The text in the first few pages sometimes suffers from lack of precision. For example, on page 2, the text reads ""For example, cells of the vertebrate brain require extensive microtubule networks for migration..." The word "migration" seems incorrect. This is minor, but the point remains that this section of the text seemed less well-edited than the rest.

2. The first section of the Methods should be reorganized to make it easier to read and follow. In particular, it seems illogical to have plasmid construction details in a section titled "yeast manipulation and culturing."

3. The authors seem a bit surprised that cells maintain the soluble tubulin pool "at the expense of microtubule polymers" (page 16), but this behavior is an expect outcome of polymer polymerization behavior and the existence of critical concentrations (biology could potentially come up with a way around it, but that would make additional complexity). Adding some explanation to this regard would be helpful.

4. Figures:
a. Figure 1B: I believe that there is a difference between WT and tub1delta, and the authors present solid quantitative data to this regard in Figure 1C, but this figure is not convincing - it appears to be more of a cartoon than data. Where are the data points? Where are the error bars? How do we know that this is not simply sample to sample variation? If the data are noisy, then this is something that the reader needs to see to help them interpret their own experiments.
b. Figure 1C: Perhaps it is just my personal preference to avoid unnecessary normalization, but why is the doubling time normalized here? I found it distracting. Also, the authors might want to check to make sure that they have described this experiment correctly. The Methods say that the experiment was done in YPD media, the combination of the reported doubling time (found later in the paper) and the OD at saturation look more like minimal media to me.

Reviewer #3 (Comments to the Authors (Required)):

This manuscript from Wethekam and Moore addresses a central problem in the regulation of microtubule cytoskeleton function. Namely, how the overall level, and relative level of alpha and beta tubulin subunits are controlled in vivo. It has long been known that excess beta tubulin is toxic to cells, and that excess alpha tubulin is not as toxic. In mammalian cells, beta tubulin levels are known to be auto-regulated such that excessive beta tubulin protein promotes degradation of mRNA, thus diminishing production of new beta subunits. In the current manuscript, Wethekam and Moore take a genetic based approach in yeast to examine how cells respond to excess alpha and/or beta tubulin. A strength of the study is that it allows controlled levels of alpha relative to beta tubulin subunits. They also employ strong overexpression to examine extreme super-stochiometric scenarios. In general, the authors find that excess alpha tubulin is tolerated much better than even a relatively small excess of beta tubulin. While excess alpha is relatively benign, excess beta disrupts microtubules, generates more persistent forms of what the authors call "tubulin assemblies," and decreases cell fitness. Thus, excess alpha is much healthier than excess beta tubulin. The authors then show that strong overexpression of alpha tubulin is reduced after 24 hours, suggesting that cells have a mechanism to reduce excess alpha tubulin expression. A similar mechanism appears to be lacking for beta tubulin. The manuscript present convincing data that excess alpha tubulin is better tolerated than beta tubulin, and that cells normally maintain excess alpha to both stoichiometrically pair with beta and to avoid the toxic effects of excess beta.

While the central tenants of this manuscript are sound, there are relatively significant issues with experimental techniques, approaches, and interpretations and/or conclusions that need to be addressed prior to publication.

1) It is a very interesting finding that alpha tubulin levels appear to equilibrate after overexpression but beta tubulin levels do not. Considering the title and conclusions of this manuscript, to generalize the study's findings the manuscript would be strengthened if both alpha-tubulin isotypes were tested.

2) Cell lysates and Western blots

Several conclusions are based on the western blots quantified in Fig 2G and related experiments. The cell types in 2G each display 3-4-fold differences in measured protein levels for alpha and beta subunits. Do repetitious cultures of the same strain actually have 3-4-fold differences in tubulin levels? Is this variability due to culture conditions? Lysis/solubilization efficiency? Western blotting? It seems like this variability may be necessitating the graph style that shows each data point delineated with its related alpha/beat measurement. It should be carefully considered by the authors and presented to the readers how this variability impacts accuracy.

Related to Fig 2E and 2F the number of tubulin molecules calculated per cell for each of the cell number samples should be made clearer. In Fig S1, the increasing cell number samples are simply shown at their values on the linear regression line made as the standard curve, which is not very informative. The purified tubulin curve is essentially linear. But it is not readily apparent whether the cell lysate tubulin readout is also linear with cell number, and it appears to possibly be not very linear. Is the lysate method less efficient with increasing cell number? Or the blot less efficient with excess surrounding cellular proteins of similar weight? As with purified tubulin, the number of tubulins calculated from the curve should be graphed against the number of cells. If the relationship is not linear, then why combine and use 4 values if they are non-linear? Would it not be more accurate to use a single, consistent value in the most linear range? But much better to use a lower range of cells if it provides a linear cell number/tubulin molecule response?

Can the authors address what may be going on with the beta tubulin heterozygous diploid or alpha/beta heterozygous strains in Fig 2H? There is a 5-fold difference in the measured alpha/beta tubulin ratios between repetitions. Is this experimental error? Or could reduced beta tubulin subunits have a more complex effect than the model proposes?

3) Soluble tubulin subunits

The text consistently refers to the soluble tubulin pool, even with regard to individual alpha and beta subunits. Yet, the study uses a cell lysate preparation method that is highly denaturing (boiling in detergent, with a 'gentle' 6,000xg spin for 3 minutes after denaturing), and all extracted tubulin polypeptides are considered soluble in the cell. It seems an aggregated protein would likely end up in the lysate using this method. In the cell, folded, soluble beta tubulin subunits likely behave differently than partially folded ones associated with folding cofactors, as well as assemblies and/or aggregates of incompletely folded subunits and complexes with folding cofactors. This needs clarification and should be carefully considered in the interpretations and conclusions.

This description and interpretation of soluble tubulin is used throughout. As an example, in the conclusions it is stated "We find that the soluble tubulin pool contains ~25% more α- tubulin than β-tubulin and there are distinct consequences when the normal ratio of α- to β-tubulin is disrupted." This would imply that applying a cell lysis prepared under non-denaturing conditions to an SEC column would show the tubulin heterodimer peak at 110kD and another 25% of alpha-tubulin in a 55kD peak without beta? While fascinating, such a conclusion is far from certain based on the lysate conditions and data presented in this study.

4) Tubulin assemblies: The approach and text does not address whether excess/super-stochiometric subunits are folded, aggregated, partially folded, in complex with folding cofactors, etc. In the denatured lysates they are all described as soluble, but it's not clear how this relates to their original status in the cell. The original status of the subunits has large implications for the structures and effects observed in vivo.

The tubulin assemblies are discussed in the results section as containing the overexpressed subunit as a soluble component along with normal tubulin heterodimers. It is mainly in the discussion where the idea that they may contain potentially aberrant subunits in complex with folding cofactors is addressed. It seems equally, or perhaps more likely, that they may be aggregates of folding intermediates alone or with cofactors resulting from the lack of a complementary subunit. This should be more clearly discussed, particularly considering the use of denaturing lysates. On the other hand, the idea of 'assemblies' could be strengthened by examining whether chaperonin and/or folding cofactors are absent from the structures.

5) The statement at the beginning of the description of Fig 5 data does not seem accurate: "when α-tubulin expression is increased cells can readily equilibrate the newly expressed protein with existing tubulin to maintain the pool of αβ-tubulin heterodimers in the cell, but increasing β-tubulin expression destroys microtubules and creates a toxic accumulation of β-tubulin protein (Figure 2G; 3)." The data in Fig. 2G does not show any increase in alpha or beta tubulin. When there is a gene excess of one subunit, that subunit is not reduced or equilibrated at the protein level but results in a 2-fold increase in ratio (2G-H). The increase in beta shown in Fig 3, while detrimental, is not comparable to the extent of excess shown in Fig 2. This statement is

either not supported and/or not explained clearly:

6) A key observation is that: "After 24 hours of induction, GFP-Tub3 levels decreased to nearly match the level of Tub1that we measured prior to galactose-induction, while Tub1 was decreased to less than 0.5x of pre-induction levels (Figure 5F)." It would be very useful if the authors indicate whether (and where) these changes are statistically significant or not, particularly for GFP-Tub3 at 2, 3, and 24 hours.

7) With the statement, "The consistent level of β-tubulin stands in contrast to the loss of Tub1 and indicates that loss of Tub1 is not attributable to titration of protein levels through cell division." Without providing more data, it seems the default assumption must be that beta expression is constant and will find an alpha partner. If tub3 is overexpressed, it will outcompete tub1 for binding to beta. Thus, the population of heterodimers will over time become enriched for tub3 over tub1. The free tub1 that has been outcompeted may be less stable and this could explain how the levels of tub1 decrease over time. This effect would be similar in concept to a titration of tub1 levels through continued tub2 expression and tub1 overexpression during cell division.

Similarly stated in the text, "When protein levels of a new α-tubulin isotype increase, the other α-tubulin isotype is gradually lost from the cell until total α-tubulin returns to a predetermined set point." Is this occurring from a phenomenon like the one above? Are these cells / cultures growing during this time or are they stationary/arrested? If they indeed double during the assay then half or more of the cells are new and it cannot be considered that an alpha tubulin isotype is gradually lost from that cell.

8) Changes in tubulin subunit levels

The following statement is unclear and does not readily appear to be supported by the data presented: "However, loss of α- or β-tubulin genes has little effect on the amount of corresponding tubulin detected in the soluble pool (Figure 2)." First, it seems the reader must deduce the average levels for each subunit/strain from the individual data points presented in Fig. 2G. Then, Fig 2H shows only the ratios. From this, in Fig. 2G it appears that the level of corresponding subunit does change (it is reduced) in the deletion strains. It would help readers to report the average values for each subunit/strain.

In the conclusion it states, "Overall, this supports a model in which cells maintain a relatively constant concentration of α- and β-tubulin in the soluble pool, and undersupplying tubulin by decreasing α- or β-tubulin gene copy number primarily affects the microtubule polymers." As a reader it is hard to see how this is clearly supported. Here is another case where it would be helpful to plot (or table) the average molecules per cell determined in Fig. 2G. Rather than consistent levels, it seems like reducing the alpha gene(s) causes alpha tubulin molecules to drop from 23,000 to 15,000 and removing a beta gene causes a drop from perhaps 19,000 to 11,000. It's hard to discern the significance of the authors' statement/conclusion in the current form.

Additionally, in Fig. 2G the level of alpha subunit indeed goes down after an alpha gene is lost, but it appears that beta subunits also go up. Averages of the genotypes/conditions would help to clearly illustrate what this data shows and what the authors wish to conclude.

9) With regard to the statement, "In contrast to α-tubulin, we propose that super-stoichiometric β-tubulin forms 'dead-end' assemblies that exhibit very slow exchange and are not readily targeted for degradation (Figure 6)." It does seem like the beta structures are not readily targeted for degradation. However, it also appears the data presented does not clearly address whether or not the super-stoichiometric beta subunits exhibit very low exchange.

10) It should be considered whether a 24 hour adjustment in the level of alpha tubulin is truly rapid, given the scale of yeast replication. It appears that 24 hours would span at least 10 doublings.

Minor comments:

The authors state, "At one hour of galactose induction, β-tubulin is increased to 2x the level measured in uninduced control cells and nearly all cells fail to form colonies (Figure 3B-D)." Although the graph axis alludes to the fact, the text and/or methods should make clear whether the cell is placed immediately onto Glucose plates or Galactose plates for colony formation.

The manuscript states, "We conclude that gene copy number for both α- and β-tubulin is important for heterodimer activity, but the lower rates observed in TUB2 heterozygotes suggest that β-tubulin may be limiting for polymerization rate." The use of the phrase 'heterodimer activity' is not clear. Are there fewer of them formed? Or are they formed but they are less active? Similarly, is beta is limiting for polymerization, or for heterodimer formation and thus polymerization? This concept is better phrased in discussion: "in yeast β-tubulin is limiting for the heterodimer state."

This statement should not be in the results section: "These results indicate that GFP-Tub3-containing assemblies may represent transient stores of α-tubulin en route to heterodimer formation and/or microtubule polymerization." While it is an intriguing thought, it is pure speculation at the point it is stated. These assemblies could also be aggregates that will eventually be degraded. Since protein levels are reduced hours later there is either lower transcription, lower translation, and/or more protein degradation. They could also be formed from clogged up folding pathway intermediates that eventually work out or are degraded when overall expression / protein levels decrease. Later in the discussion this idea is more thoroughly discussed, but this limited

statement should not be in the results without full consideration.

Our responses to each reviewer's points are provided in the boxed text, below.

Reviewer #1 (Comments to the Authors (Required)):

Wethekam and Moore, Asymmetric requirement for α-tubulin over β-tubulin.

A tubulin subunit is a heterodimer of αβ-tubulin. However, there are 2 alpha-tubulin genes but only 1 beta-tubulin gene in the budding yeast. The authors set out to determine the functional significance of this asymmetry in the context of microtubule dynamics and spindle assembly. They genetically alter the expression level of the different tubulin genes, then monitor consequences of microtubules and spindles. The results suggest that budding yeast is more sensitive to the level of β-tubulin for proper function.

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It has been previously and well-established that budding yeast TUB1 (alpha) and TUB2 (beta) is essential, and Tub3 (alpha) in non-essential. The current work, while introducing the term "tubulin assemblies" to describe aggregates of non-functional tubulins, and establishing concentrations of alpha and beta tubulins in budding yeast, does not shed further light on known observations.

I do not recommend publication in the JCB.

Minor comments:

Fig 1D: Some mutant colonies grow better than wild-type, why?

The larger colonies of mutant cells in Figure 1D is primarily due to the growth conditions in the assay. In this experiment, cells are plated in a 10-fold dilution series going from left to right so that the spots at far right contain <50 individual cells that were originally transferred to the plate. As these cells divide and form colonies, the size of the colonies is limited by the fitness of the cells and by the availability of nutrients in that region of the plate. Cells and colonies that are less crowded with neighbors have access to more nutrients and will form larger individual colonies than the cells from the same original cultures in the

more crowded spots to their left, which more rapidly deplete the concentration of nutrients in the plate.

As the reviewer observes, particularly the *tub1*Δ heterozygote and to a lesser extent the *tub3*Δ heterozygote strains in Figure 1D each show a few larger colonies on the right side of the plate containing 5µg/mL benomyl and the plate grown at 15ºC. We speculate that these larger colonies may represent a small number of suppressor isolates that emerged in these populations of heterozygotes. The nature of these suppressors is not obvious, but it is possible that these are isolates that have either increased α-tubulin expression or decreased β-tubulin expression, creating a fitness benefit. Our speculation is based on a previous study, where we found that isogenic haploid strains expressing a mutant allele of *TUB1* α-tubulin showed a similar frequency of suppressors, and that these suppressors often showed higher levels of *TUB3* expression. For the few larger colonies found in Figure 1D of the current manuscript we have not explored the source of this variation. Since the size of these colonies is exacerbated by the time that they are allowed to grow in the incubator, in the revised Figure 1D we have replaced the images that previously showed plates grown at 30ºC for 3 days with plates grown at 30ºC for 2 days. Hopefully this makes the data more clear and the few large colonies less distracting.

Fig 1G: How are "coefficient of variation" measure?

We thank the reviewer for catching this oversight. We have now added a description of how we calculated coefficient of variation for pre-anaphase spindle lengths. This can be found in the Methods section on page 20, under the sub-title “Pre-anaphase spindle measurements”:

“To calculate the coefficient of variation, the standard deviation of pre-anaphase spindle length measurements for an individual cell was divided by its mean length value.”

Fig 2A: A few time-lapsed images would suffice to show growth or shrinkage.

We appreciate the reviewer’s point that a few example images is often preferrable, however in this case we feel strongly that the data are best represented by an example image in Figure 2A that shows the Bik1-3GFP signal that we measure in the assay, and the traces in 2B that show the full set of measurements. The data sets for these microtubule dynamics assays contain 120 Z-series per cell and the full data set is necessary to identify reliable rates and transition frequencies. Therefore, a few images do not faithfully represent the data.

Fig 2G and 2H: For some measurements, half of the population show the opposite trend. In this context of opposite results in the same measurement, can one make a clear conclusion?

The reviewer makes an important point and we have made significant efforts to address this concern in the revised manuscript. To summarize our efforts, we re-examined all of the western blotting data used in Figure 2G and H, and found that variation arising from some of our western blots was leading to aberrantly high or low estimates of tubulin concentrations in some cases. We addressed this by developing a 'quality control' protocol where a coefficient of determination was calculated for each western blot of a cell lysate dilution series. We used this measure to identify data points in the dilution series, typically at the lower or upper ends of the series, that did not exhibit a strong linear relationship with the rest of the series (i.e., their inclusion gave an $r^2 < 0.75$). We removed these points to improve the quality of our data so that the remaining series always contained at least 3 data points and an $r^2 > 0.75$. These complete data sets are now included as Supplemental Figures S2-S3. For further details on this point, please see our response below to Reviewer 3, point 2.

The data that we now report in Figure 2G and H show consistent trends for wild type, tub1Δ heterozygotes, and tub2Δ heterozygotes. However, the tub1Δ tub2Δ double heterozygotes still show a mixed set of results with trends in both directions. Because this is only observed for this genotype, we suspect that this variation arises from biological variability in the double heterozygote. Consequently, we avoid making a strong conclusion about the ratio of α- to β-tubulin in these cells.

Fig 3E and 4D: Can one define non-functional tubulin aggregates as "tubulin assemblies" to probe further?

We agree that the composition, structure and functional consequences of tubulin assemblies are interesting questions that are not completely answered in the current manuscript. We are currently working on answering these questions as part of a separate project.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript uses a combination of molecular genetics, quantitative light microscopy, and quantitative western blots to address the question of how the levels of alpha and beta tubulin are (separately) regulated in yeast. The authors conclude that alpha tubulin levels are regulated and kept at levels higher than beta tubulin, in part by having multiple alpha tubulin genes. As part of this work, they show that an excess of beta tubulin is highly detrimental, but excess alpha tubulin has little effect, a conclusion that they reach through a series of complementary experiments.

Overall, this is solid paper: carefully designed, conducted, interpreted, and presented. It uses appropriate approaches to provide a mechanistic answer to an important biological problem. This Discussion is interesting and does a good job of placing this work in the context of other work. I have only two concerns. One is that there are some minor writing issues, which I discuss below. My more significant hesitation is that the mechanism that the authors identify seems likely to be specific to yeast, or at least not shared with mammals, as the authors themselves discuss in the Discussion. I personally don't think that this is a problem because many aspects of cell biology that have seemed different in yeast have turned out to provide insight into mammalian and other systems (the actin/myosin-based nature of membrane transport in yeast is one example). In my opinion, the Discussion addresses this issue sufficiently (I would have been much more concerned if the Discussion had not addressed this issue head-on).

Writing:
1. The quality of writing in the Abstract and Introduction is not quite up to that in the rest of the paper. Here are a few examples from the first two pages (this list is not exhaustive)

a. The order in which ideas are presented in the Abstract is confusing. For example, the first description of an observation states, "We find that α-tubulin gene copy number is important for maintaining an excess α-tubulin protein compared to β-tubulin protein and preventing accumulation of super-stoichiometric β-tubulin." It seems to this reader that this statement (and others following) would make more sense if the authors first stated that they observe that α-tubulin is maintained in an excess. Regardless of how the authors edit the abstract, I do think that it needs some work.

We have now re-written the abstract as suggested by the reviewer. We believe this has improved the clarity of the abstract. We thank the reviewer for this suggestion.

b. The abstract should include the fact that the observations were made in S. cerevisiae.

We now state in the abstract that our investigation was carried out in S. cerevisiae.

c. The text in the first few pages sometimes suffers from lack of precision. For example, on page 2, the text reads ""For example, cells of the vertebrate brain require extensive microtubule networks for migration..." The word "migration" seems incorrect. This is minor, but the point remains that this section of the text seemed less well-edited than the rest.

We have now re-written this portion of the introduction on page 2 and include citations to papers that help illustrate our points. The sentence now reads as follows:

For example, neurons of the vertebrate brain require extensive microtubule networks for migration, axon formation and extension during development; and trafficking components to and from synapses in mature neurons (Alfadil and Bradke, 2022; Aiken and Holzbaur, 2021).

2. The first section of the Methods should be reorganized to make it easier to read and follow. In particular, it seems illogical to have plasmid construction details in a section titled "yeast manipulation and culturing."

We have made this suggested change in the revised manuscript, separating the first section of the Methods into a section on “Yeast manipulation and culturing” and a separate section on “Plasmid construction”. We thank the reviewer for this suggestion.

3. The authors seem a bit surprised that cells maintain the soluble tubulin pool "at the expense of microtubule polymers" (page 16), but this behavior is an expect outcome of polymer polymerization behavior and the existence of critical concentrations (biology could potentially come up with a way around it, but that would make additional complexity). Adding some explanation to this regard would be helpful.

We share the reviewer’s expectation of the relationship between the soluble pool of tubulin and the pool in microtubule polymers. We merely point this out in the discussion because our results are consistent with that conventional model. To make this expectation more clear, we have added the following sentence to the second paragraph of the discussion on page 14:

Undersupplying tubulin by decreasing α- or β-tubulin gene copy number affects the length and polymerization rate of microtubule polymers, consistent with the expected equilibrium between soluble tubulin and microtubules.

4. Figures:
a. Figure 1B: I believe that there is a difference between WT and tub1delta, and the authors present solid quantitative data to this regard in Figure 1C, but this figure is not convincing - it appears to be more of a cartoon than data. Where are the data points? Where are the error bars? How do we know that this is not simply sample to sample variation? If the data are noisy, then this is something that the reader needs to see to help them interpret their own experiments.

In the revised manuscript we have amended Figure 1B to show the mean values as lines with shading added to indicate 95% confidence intervals based on the technical replicates used in this experiment.

b. Figure 1C: Perhaps it is just my personal preference to avoid unnecessary normalization, but why is the doubling time normalized here? I found it distracting. Also, the authors might want to check to make sure that they have described this experiment correctly. The Methods say that the experiment was done in YPD media, the combination of the reported doubling time (found later in the paper) and the OD at saturation look more like minimal media to me.

We report normalized doubling times in Figure 1C because these data encompass sets of experiments that were conducted over several years on two different plate reader instruments. Our initial experiments were conducted on a Cytation 3 instrument that is shared equipment in our department, but we subsequently bought an Epoch 2 instrument for our own lab that was used for later experiments. We found that the doubling time data collected on these two instruments is slightly different, even for technical replicates of isogenic strains. Therefore we normalized the data in each experiment to internal wild-type controls, and report those normalized data in Figure 1C. We have now added these details on the two different instruments in the Materials and Methods section.

With regard to the description of the experiment, we confirmed that these experiments were done in YPD media. We suspect that the lower OD values seen at saturation in our experiments may be attributable to the volume (200µL) and aeration in our experiment, compared to alternative methods using larger culture volumes with different aeration.

Reviewer #3 (Comments to the Authors (Required)):

This manuscript from Wethekam and Moore addresses a central problem in the regulation of microtubule cytoskeleton function. Namely, how the overall level, and relative level of alpha and beta tubulin subunits are controlled in vivo. It has long been known that excess beta tubulin is toxic to cells, and that excess alpha tubulin is not as toxic. In mammalian cells, beta tubulin levels are known to be auto-regulated such that excessive beta tubulin protein promotes degradation of mRNA, thus diminishing production of new beta subunits. In the current manuscript, Wethekam and Moore take a genetic based approach in yeast to examine how cells respond to excess alpha and/or beta tubulin. A strength of the study is that it allows controlled levels of alpha relative to beta tubulin subunits. They also employ strong overexpression to examine extreme super-stochiometric scenarios.
In general, the authors find that excess alpha tubulin is tolerated much better than even a relatively small excess of beta tubulin. While excess alpha is relatively benign, excess beta disrupts microtubules, generates more persistent forms of what the authors call "tubulin assemblies," and decreases cell fitness. Thus, excess alpha is much healthier than excess beta tubulin. The authors then show that strong overexpression of alpha tubulin is reduced after 24 hours, suggesting that cells have a mechanism to reduce excess alpha tubulin expression. A similar mechanism appears to be lacking for beta tubulin. The manuscript present

convincing data that excess alpha tubulin is better tolerated than beta tubulin, and that cells normally maintain excess alpha to both stoichiometrically pair with beta and to avoid the toxic effects of excess beta.
While the central tenants of this manuscript are sound, there are relatively significant issues with experimental techniques, approaches, and interpretations and/or conclusions that need to be addressed prior to publication.

1) It is a very interesting finding that alpha tubulin levels appear to equilibrate after overexpression but beta tubulin levels do not. Considering the title and conclusions of this manuscript, to generalize the study's findings the manuscript would be strengthened if both alpha-tubulin isotypes were tested.

We agree that this is an important point since the α-tubulin isotypes could in principle have different expression and/or degradation kinetics. In the revised manuscript we have now added a new experiment that tests this prediction. We repeated our experiment inducing high levels of TUB1 expression from a galactose-inducible promoter and measured tubulin protein levels after 24 hours of induction. We show in a revised Figure 4B that after 24 hours of Tub1 induction, α-tubulin returns to levels similar to those measured prior to induction. This indicates that both α-tubulin isotypes equilibrate after overexpression. We thank the reviewer for this suggestion.

2) Cell lysates and Western blots

Several conclusions are based on the western blots quantified in Fig 2G and related experiments. The cell types in 2G each display 3-4-fold differences in measured protein levels for alpha and beta subunits. Do repetitious cultures of the same strain actually have 3-4-fold differences in tubulin levels? Is this variability due to culture conditions? Lysis/solubilization efficiency? Western blotting? It seems like this variability may be necessitating the graph style that shows each data point delineated with its related alpha/beat measurement. It should be carefully considered by the authors and presented to the readers how this variability impacts accuracy.

Related to Fig 2E and 2F the number of tubulin molecules calculated per cell for each of the cell number samples should be made clearer. In Fig S1, the increasing cell number samples are simply shown at their values on the linear regression line made as the standard curve, which is not very informative. The purified tubulin curve is essentially linear. But it is not readily apparent whether the cell lysate tubulin readout is also linear with cell number, and it appears to possibly be not very linear. Is the lysate method less efficient with increasing cell number? Or the blot less efficient with excess surrounding cellular proteins of similar weight? As with purified tubulin, the number of tubulins calculated from the curve should be

graphed against the number of cells. If the relationship is not linear, then why combine and use 4 values if they are non-linear? Would it not be more accurate to use a single, consistent value in the most linear range? But much better to use a lower range of cells if it provides a linear cell number/tubulin molecule response?

We thank the reviewer for these suggestions an agree that the values reported in the original Figure 2G and H suggested some major source or sources of variability. In the revised manuscript we have made serious efforts to scrutinize potential sources of variability and apply more stringent analysis methods to improve confidence in our results.

Culture conditions.

Our data set includes data collected from cells cultured to mid-log phase under the same conditions. We did this mindful of the possibility that tubulin levels may change in rapidly-dividing vs stationary phase cells. To assess variation in cell number and viability, we counted and plated dilutions of cells from the same cultures used to make each lysate used in our westerns. This confirmed the number of cells per sample and the viability of those cells. We now report the fraction of those cells that formed colonies as Figure S2A.

Lysis efficiency

To assess variability that may arise from the cell lysis method, we compared the signal of Zwf1/G6PD from western blots of cell lysate dilution series across experiments. Our reasoning is that variation in lysis efficiency should create variable signal values for Zwf1/G6PD per number of cells in the lysate. However, this is not the case. We find that Zwf1/G6PD signal intensity per number of cells in the sample is highly consistent across experiments. These data are now presented for each genotype analyzed in the new Figures S2-5.

Western blotting

As described in response to Reviewer #1, above, we assessed variability from western blotting by re-examining all of the western blotting data used in Figure 2G and H. We found that many of the experimental data sets that were used to create the results in Figure 2G and H of the original manuscript gave low values for coefficient of determination; i.e. the signal for α or β-tubulin was not strongly correlated to the number of cells in the lysate sample. Having identified this as a major source of variation, we applied a more stringent quality control method to identify low-confidence data points and remove them from the data set. For dilution series of cell lysates that gave coefficient of determination values lower than r^2=0.75, we identified data points in the dilution series, typically at the lower or upper ends of the series, that did not exhibit a strong linear relationship with the rest of the series. We removed these points so that the remaining series always contained at least 3 data points and an $r^2 > 0.75$. All of our measurements for Figure 2G and H are now analyzed in this way, and the full data sets depicting signal intensity vs cell number in each sample are shown in Figures S2-3, along with the corresponding r^2 values. Applying this more stringent

analysis method decreased variation in our molecule/cell calculations and in our estimated ratios of α to β-tubulin.

Can the authors address what may be going on with the beta tubulin heterozygous diploid or alpha/beta heterozygous strains in Fig 2H? There is a 5-fold difference in the measured alpha/beta tubulin ratios between repetitions. Is this experimental error? Or could reduced beta tubulin subunits have a more complex effect than the model proposes?

As stated above in response to Reviewer #1, applying our new quality control analysis greatly diminished the variability in the tub2Δ heterozygote results. However, the tub1Δ tub2Δ double heterozygotes still show a mixed set of results for ratios across our replicate experiments, with trends in both directions. Because this is only observed for this genotype, we suspect that this variation arises from biological variability in the double heterozygote. We would note that we find variable results across two independent biological replicates for the double heterozygote. The source of this variability is not clear. Consequently, we avoid making a strong conclusion about the ratio of α- to β-tubulin in these cells.

3) Soluble tubulin subunits

The text consistently refers to the soluble tubulin pool, even with regard to individual alpha and beta subunits. Yet, the study uses a cell lysate preparation method that is highly denaturing (boiling in detergent, with a 'gentle' 6,000xg spin for 3 minutes after denaturing), and all extracted tubulin polypeptides are considered soluble in the cell. It seems an aggregated protein would likely end up in the lysate using this method. In the cell, folded, soluble beta tubulin subunits likely behave differently than partially folded ones associated with folding cofactors, as well as assemblies and/or aggregates of incompletely folded subunits and complexes with folding cofactors. This needs clarification and should be carefully considered in the interpretations and conclusions.

We agree with the reviewer that our denaturing method of cell lysis is not necessarily yielding just the soluble pool of tubulin on the cell. We have changed our wording in the revised manuscript to avoid referring to the tubulin in these samples as the soluble pool. We now describe this method as denaturing and simply refer to the tubulin in these samples as the tubulin in the cell lysate created by this method.

This description and interpretation of soluble tubulin is used throughout. As an example, in the conclusions it is stated "We find that the soluble tubulin pool contains ~25% more α-tubulin than β-tubulin and there are distinct consequences when the normal ratio of α- to β-tubulin is disrupted." This would imply that applying a cell lysis prepared under non-

denaturing conditions to an SEC column would show the tubulin heterodimer peak at 110kD and another 25% of alpha-tubulin in a 55kD peak without beta? While fascinating, such a conclusion is far from certain based on the lysate conditions and data presented in this study.

We found this suggestion to be very intriguing. To explore it, we conducted new experiments to create protein lysates by an alternative method – high pressure cell rupture in a buffer that maintains tubulin solubility. We clarified these lysates through a series of 6,000 x g and 100,000 x g spins, and ran quantitative western blots of the clarified lysates. Our results, which are now reported in Figure S1, indicate that the level of α-tubulin in these soluble lysates is much closer to the level of β-tubulin, and does not show the same excess of α-tubulin that we see in the samples prepared by denaturing lysis. This suggests that the excess of α-tubulin may not be in the soluble fraction and may only be found in lysates prepared under denaturing conditions. The excess α-tubulin could be in an aggregate or assembly state, or in complex with chaperones or TBCs.

4) Tubulin assemblies: The approach and text does not address whether excess/super-stochiometric subunits are folded, aggregated, partially folded, in complex with folding cofactors, etc. In the denatured lysates they are all described as soluble, but it's not clear how this relates to their original status in the cell. The original status of the subunits has large implications for the structures and effects observed in vivo.

The tubulin assemblies are discussed in the results section as containing the overexpressed subunit as a soluble component along with normal tubulin heterodimers. It is mainly in the discussion where the idea that they may contain potentially aberrant subunits in complex with folding cofactors is addressed. It seems equally, or perhaps more likely, that they may be aggregates of folding intermediates alone or with cofactors resulting from the lack of a complementary subunit. This should be more clearly discussed, particularly considering the use of denaturing lysates. On the other hand, the idea of 'assemblies' could be strengthened by examining whether chaperonin and/or folding cofactors are absent from the structures.

We agree with the reviewer that the details of tubulin assemblies for α or β-tubulin are not completely resolved. The results from our new experiments (described in the previous point) creating soluble lysates by an alternative method could be consistent with the excess tubulin in an aggregate or other complex form.

We sought to test the prediction that assemblies are complexes of tubulin with TBCs. Although TBCs have not been previously shown to co-localize with tubulin-containing structures in cells, it is possible that they may be sufficiently recruited to tubulin assemblies to allow for visualization. We tested this by fusing mNeonGreen to the C-termini of the TBCs Cin1, Cin2 and Cin4 or fusing tdTomato to the C-termini of the TBC, Pac2, at their native chromosomal loci, and using spinning disk confocal microscopy to localize each fusion

under conditions where α or β-tubulin is overexpressed. We found no evidence of co-localization for any of the fusions in any of the conditions tested. Furthermore, we tested the function of each fusion in growth assays with challenged microtubules and confirmed that none of the tags strongly affected TBC function. Although we cannot rule out transient interactions, these results do not support the hypothesis tubulin assemblies are aggregates of folding intermediates in complex with TBCs. Since these data are negative and TBC localization remains undescribed, we decided not to include these results in the manuscript.

To test whether excess tubulin is in complex with chaperonin, we tested the prediction that α or β-tubulin overexpression might occupy chaperonin and block or slow the biogenesis of actin, another chaperonin client. We did this by visualizing actin cables and patches in cells experiencing high levels of tubulin over expression. We found no evidence of actin loss or disruption. These results do not support the hypothesis that tubulin assemblies are folding intermediates in complex with chaperonin. Since these results are negative we decided not to include them in the manuscript.

The composition, structure and functional impact of tubulin assemblies remain important but unanswered questions. We feel that answering these questions is beyond the scope of the current manuscript, and are currently exploring them in a follow-up study.

5) The statement at the beginning of the description of Fig 5 data does not seem accurate: "when α-tubulin expression is increased cells can readily equilibrate the newly expressed protein with existing tubulin to maintain the pool of αβ-tubulin heterodimers in the cell, but increasing β-tubulin expression destroys microtubules and creates a toxic accumulation of β-tubulin protein (Figure 2G; 3)." The data in Fig. 2G does not show any increase in alpha or beta tubulin. When there is a gene excess of one subunit, that subunit is not reduced or equilibrated at the protein level but results in a 2-fold increase in ratio (2G-H). The increase in beta shown in Fig 3, while detrimental, is not comparable to the extent of excess shown in Fig 2. This statement is either not supported and/or not explained clearly:

We agree with the reviewer that this statement describes the results from the overexpression experiments in Figures 3 and 4, and not the gene loss experiments in Figure 2G. We have removed the call out of Figure 2G from this sentence to clarify that point.

However, we feel that the overexpression experiments are revealing effects that are similar to those seen in the gene loss experiments. We elaborate on this point in the last paragraph on page 16 of the discussion. We point out that when one copy of TUB1 is knocked out, creating a gene excess for β-tubulin, β-tubulin protein levels are higher than in wild-type cells. But when one copy of TUB2 is knocked out, creating an excess of α-tubulin, we do not see high levels of α-tubulin protein. In that case, both α- and β-tubulin levels are decreased, consistent with the equilibration of excess α-tubulin seen in our overexpression

experiments. We have added the estimated values for molecules per cell to Table 1 to make this clearly available to the reader.

6) A key observation is that: "After 24 hours of induction, GFP-Tub3 levels decreased to nearly match the level of Tub1that we measured prior to galactose-induction, while Tub1 was decreased to less than 0.5x of pre-induction levels (Figure 5F)." It would be very useful if the authors indicate whether (and where) these changes are statistically significant or not, particularly for GFP-Tub3 at 2, 3, and 24 hours.

We have now performed statistical analysis of the levels of GFP-Tub3 at 2,3, and 24 hrs. We find that the decline in GFP-Tub3 levels from 3 to 24 hours of induction is significant, based on t-test ($p = 0.041$). We now indicate the values from these statistical tests in Figure 5F.

7) With the statement, "The consistent level of β-tubulin stands in contrast to the loss of Tub1 and indicates that loss of Tub1 is not attributable to titration of protein levels through cell division." Without providing more data, it seems the default assumption must be that beta expression is constant and will find an alpha partner. If tub3 is overexpressed, it will outcompete tub1 for binding to beta. Thus, the population of heterodimers will over time become enriched for tub3 over tub1. The free tub1 that has been outcompeted may be less stable and this could explain how the levels of tub1 decrease over time. This effect would be similar in concept to a titration of tub1 levels through continued tub2 expression and tub1 overexpression during cell division.

Yes, we agree with the reviewer's interpretation. Our model is that α-tubulin turnover is driven by competition for a β-tubulin partner combined with degradation of an unstable, partner-less α monomer. We currently favor this competition model for explaining the gradual loss of one α isotype when a different isotype is expressed at high levels; e.g., Tub1 loss when Tub3 is overexpressed. There are alternative possibilities, such as downregulation of Tub1 biogenesis, but we do not have data supporting that possibility.

Similarly stated in the text, "When protein levels of a new α-tubulin isotype increase, the other α-tubulin isotype is gradually lost from the cell until total α-tubulin returns to a predetermined set point." Is this occurring from a phenomenon like the one above? Are these cells / cultures growing during this time or are they stationary/arrested? If they indeed double during the assay then half or more of the cells are new and it cannot be considered that an alpha tubulin isotype is gradually lost from that cell.

How cell division contributes to the equilibration of α-tubulin levels is important to consider. Indeed, the cells are doubling during the GFP-Tub3 induction experiment. We

have not measured the rate of proliferation, but we know this qualitatively from the increasing density of the cultures. So we must assume that the samples collected at later timepoints in the experiment, particularly at 24 hours post-induction, include new cells that were born while GFP-Tub3 was being induced. We also assume that roughly half of a mother cell's tubulin is inherited by each daughter due to the inheritance of one half of the mitotic spindle during cell division. This titration alone would be expected to halve the cell's tubulin levels each time it divides.

We find that β-tubulin levels are relatively constant during the GFP-Tub3 induction time course, suggesting that the production of new β combined with β degradation in daughter cells must make up for the loss that occurs during cell division. In contrast, our finding that Tub1 gradually declines during the same time course indicates that either Tub1 production is diminished or its degradation is increased when GFP-Tub3 is overexpressed. As stated above, we favor the model that Tub1 is degraded at a higher rate because elevated GFP-Tub3 outcompetes Tub1 for partnering with β-tubulin.

8) Changes in tubulin subunit levels

The following statement is unclear and does not readily appear to be supported by the data presented: "However, loss of α- or β-tubulin genes has little effect on the amount of corresponding tubulin detected in the soluble pool (Figure 2)." First, it seems the reader must deduce the average levels for each subunit/strain from the individual data points presented in Fig. 2G. Then, Fig 2H shows only the ratios. From this, in Fig. 2G it appears that the level of corresponding subunit does change (it is reduced) in the deletion strains. It would help readers to report the average values for each subunit/strain.

In the conclusion it states, "Overall, this supports a model in which cells maintain a relatively constant concentration of α- and β-tubulin in the soluble pool, and undersupplying tubulin by decreasing α- or β-tubulin gene copy number primarily affects the microtubule polymers." As a reader it is hard to see how this is clearly supported. Here is another case where it would be helpful to plot (or table) the average molecules per cell determined in Fig. 2G. Rather than consistent levels, it seems like reducing the alpha gene(s) causes alpha tubulin molecules to drop from 23,000 to 15,000 and removing a beta gene causes a drop from perhaps 19,000 to 11,000. It's hard to discern the significance of the authors' statement/conclusion in the current form.

Additionally, in Fig. 2G the level of alpha subunit indeed goes down after an alpha gene is lost, but it appears that beta subunits also go up. Averages of the genotypes/conditions

would help to clearly illustrate what this data shows and what the authors wish to conclude.

We have now added columns to Table 1 that report the average number of estimated α and β-tubulin molecules per cell.

9) With regard to the statement, "In contrast to α-tubulin, we propose that super-stoichiometric β-tubulin forms 'dead-end' assemblies that exhibit very slow exchange and are not readily targeted for degradation (Figure 6)." It does seem like the beta structures are not readily targeted for degradation. However, it also appears the data presented does not clearly address whether or not the super-stoichiometric beta subunits exhibit very low exchange.

We agree that the data presented do not clearly support this proposal. We have now re-written this sentence to emphasize that this is speculation at this point:

In contrast to α-tubulin, we speculate that super-stoichiometric β-tubulin may form 'dead-end' assemblies that exhibit very slow exchange and are not readily targeted for degradation (Figure 6).

10) It should be considered whether a 24 hour adjustment in the level of alpha tubulin is truly rapid, given the scale of yeast replication. It appears that 24 hours would span at least 10 doublings.

We agree that the adjustment of α-tubulin levels that occurs over a time period of hours is not particularly rapid. We have removed this use of 'rapid' from the abstract. We do still use the term 'rapid' to describe the exchange of newly synthesized α-tubulin with the pre-existing tubulin pool, since our data suggest that this exchange occurs on the order of minutes.

Minor comments:

The authors state, "At one hour of galactose induction, β-tubulin is increased to 2x the level measured in uninduced control cells and nearly all cells fail to form colonies (Figure 3B-D)." Although the graph axis alludes to the fact, the text and/or methods should make clear whether the cell is placed immediately onto Glucose plates or Galactose plates for colony formation.

We have now added text to the Materials and Methods that clarifies this point.

The manuscript states, "We conclude that gene copy number for both α- and β-tubulin is

important for heterodimer activity, but the lower rates observed in TUB2 heterozygotes suggest that β-tubulin may be limiting for polymerization rate." The use of the phrase 'heterodimer activity' is not clear. Are there fewer of them formed? Or are they formed but they are less active? Similarly, is beta is limiting for polymerization, or for heterodimer formation and thus polymerization? This concept is better phrased in discussion: "in yeast β-tubulin is limiting for the heterodimer state."

We appreciate the reviewer's point that 'heterodimer activity' is a vague phrase. We have addressed this in the results section in the first paragraph on page 6, replaced 'heterodimer activity' with 'polymerization activity' to clearly describe the result. The sentence now reads:

"We conclude that gene copy number for both α- and β-tubulin is important for polymerization activity, but the lower rates observed in TUB2 heterozygotes suggest that β-tubulin may be limiting for polymerization activity."

This statement should not be in the results section: "These results indicate that GFP-Tub3-containing assemblies may represent transient stores of α-tubulin en route to heterodimer formation and/or microtubule polymerization." While it is an intriguing thought, it is pure speculation at the point it is stated. These assemblies could also be aggregates that will eventually be degraded. Since protein levels are reduced hours later there is either lower transcription, lower translation, and/or more protein degradation. They could also be formed from clogged up folding pathway intermediates that eventually work out or are degraded when overall expression / protein levels decrease. Later in the discussion this idea is more thoroughly discussed, but this limited statement should not be in the results without full consideration.

We take the reviewer's point here. We have re-written this sentence to simply state in the second paragraph of page 12:

"These results suggest that GFP-Tub3-containing assemblies are transient structures."

August 31, 2022

Re: JCB manuscript #202202102R

Dr. Jeffrey K Moore
University of Colorado Anschutz Medical Campus
12801 E 17th Ave
MS8108
Aurora, Colorado 80045

Dear Dr. Moore,

Thank you for submitting your revised manuscript entitled "Asymmetric requirement for α-tubulin over β-tubulin." Please accept our apologies for the delay in the processing of the reviews and thank you for your patience. The manuscript has been seen by two of the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, important issues remain that preclude acceptance at this stage.

The key concerns, expressed by Reviewer #3, are the need for improved data presentation and clarification of methods regarding the quantifications of tubulin levels as well as to tone down several conclusions that are not fully supported by the results.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that we will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Melissa Gardner, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

Overall, I think that the authors have responded well to the reviewer critiques by providing significant new data to address reviewer concerns and new text to go with these experiments, and they have also made the other suggested edits. In my opinion, the manuscript will be ready for publication after a few minor issues are addressed, all of which accomplished at the level of writing.

Minor concerns:
1) I am a bit puzzled that the authors did not respond to Review 1's major concern that "It has been previously and well-established that budding yeast TUB1 (alpha) and TUB2 (beta) is essential, and Tub3 (alpha) in non-essential. The current work, while introducing the term "tubulin assemblies" to describe aggregates of non-functional tubulins, and establishing concentrations of alpha and beta tubulins in budding yeast, does not shed further light on known observations."

In my opinion, this paper provides considerably cell biological, biochemical, and mechanistic depth to this long-established but puzzling observation. To avoid having other readers come to a conclusion similar to that of Reviewer 1, the authors could consider adding to the Discussion a more explicit statement about how their work relates to and extends beyond this earlier work.

2) The following sentence on page 15 is an improvement relative to the original, but still needs some edits: "Undersupplying tubulin by decreasing α- or β-tubulin gene copy number affects the length and polymerization rate of microtubule polymers, consistent with the expected equilibrium between soluble tubulin and microtubules." The balance between soluble and polymerized tubulin is not technically an equilibrium but a steady-state. Replacements for "equilibrium" might include "balance," "steady-state," or "relationship." Also, because people outside the microtubule field might be confused by this point, it could be helpful to provide a reference.

Reviewer #3 (Comments to the Authors (Required)):

This manuscript from Wethekam et al. takes a genetic based approach, controlling tubulin gene number in yeast, to address the central question of how overall tubulin heterodimer level, and relative level of alpha and beta tubulin subunits are controlled in vivo. The experiments are well planned and executed. While the revised manuscript does a good job of addressing most of the reviewer comments, before publication there remain some aspects of data presentation and interpretation that should be clarified. There are also instances of interpretations/conclusions that are not supported by the data presented and should be modified and/or more precisely stated.

Major comments:

Tubulin levels:

1) The authors have applied quality control approaches to the western blot data by removing data points that show decreased tubulin levels despite increasing lysate loading or overall linear regression with $r2<0.75$. This resulted in removal of some of the noisier experiments and increased linearity of the remaining points. The linearity of signal intensity is now presented in supplemental graphs. These data are then used to calculate the number of tubulin molecules per cell. As with the purified tubulin standards, the cell lysates would be expected to show a tubulin signal response that is proportional to the dilution.

Although the quality control methods increase linearity, they don't address the issue of proportionality of increase between tubulin signal/molecules and the amount of lysate/number of cells loaded. It appears that in most of the standard curves the tubulin intensity signals approximately triple from 5 to 15 ng loading. In many of the samples the tubulin signal appears to increase only a fraction (~30%) while cell number more than doubles. This gives the impression that the number of tubulin molecules calculated per cell would be far from linear across these dilutions. Rather than raw signal intensity, it would be more informative if the corresponding number of tubulins (calculated from the standard curve) is graphed against the lysate amount/number of cells.

Because these data are used to generate molecules per cell, rather than showing linear raw signal intensity, it would be much more informative if the corresponding number of tubulin molecules (calculated from the standard curve) per cell is graphed over the increase in number of cells loaded. If tubulin/cell is consistent across cell loading it would significantly strengthen the presentation. If not, it raises the question whether combining four values that may differ by 2-fold across the dilution series generates an accurate number of molecules per cell. If tubulin signal is not increasing proportionally with protein loaded, would it perhaps be more accurate to use the values calculated from the least loading? In either case it will help the reader to interpret the underlying data and resulting conclusions.

Decreasing tubulin levels over 24 hours:

2) The overexpressed alpha isotype decreases from 3 to 24 hours as much or more than the non-overexpressed alpha. This seems to show the decrease in alpha is a general response on overall alpha level. As the authors state, this could occur at multiple points in tubulin proteostasis. Alternatively, it's unclear if the cells/cultures are still in exponential phase at the 24-hour timepoint. If not, might this affect expression levels? If the cells/cultures were kept in exponential phase, can it be excluded that there is any downregulation of GAL expression over 24 hours, or that selective pressure enriched for cells that express less tubulin, e.g., the cells overexpressing alpha tubulin doubled only once in the 6-hour videos.

3) The discussion states: "The autoregulation model predicts that this increase in soluble tubulin should induce mRNA decay and thereby inhibit further translation, creating a steady state level of β-tubulin. In our experiments, β-tubulin levels continue to increase for hours without reaching steady state levels (Figure 3E-M) [this may be an incorrect callout to Fig 5H-J?]. In contrast to α-tubulin, we speculate that super-stoichiometric β-tubulin may form 'dead-end' assemblies that exhibit very slow exchange and are not readily targeted for degradation (Figure 6)." This statement overstates the data on beta tubulin levels and seemingly draws an unsupported conclusion between alpha and beta levels. The data in Fig 5I show beta levels are steady from 3-5 hours, and given the SD are likely statistically indistinguishable from 2-5 hours. Thus, they increased before 2 hours but remain at steady state until the end of the time course. Whether they remain there beyond 5 hours, increase at later time points, or perhaps decrease is not known.

4) Near the end of results on page 14 states: "This suggests that β-tubulin expression does not equilibrate like α-tubulin

expression." The total levels of alpha (5F, as well as 4B) and beta (5I) tubulin following induction look similar at 0, 2, and 3 hours. But this is where the comparison between alpha and beta ends. Beta tubulin average is slightly increased (but essentially steady state given SD) at 4 and 5 hours, but alpha tubulin is not examined in this range. Alpha tubulin is only examined at 24 hours, but beta tubulin (for technical reasons) is not examined beyond 5 hours. Unless the authors can explain something more clearly, the only difference that can be gleaned from this comparison is the decrease in Tub1p at 3 hours (Fig 5G) compared to the steady level of Tub2-438D over the same period (Fig 5J), i.e., there is no indication whether beta levels would equilibrate back to near normal levels or not.

5) The manuscript states: "Our western blots show that total α-tubulin is increased approximately two-fold at 2 hours post induction, followed by a gradual decrease that returns to preinduction levels by 24 hours (Figure 5E, F)." The 3-hour point is equal or higher than the 2-hour level, and the only next point examined is at 24 hours. There is no data showing when the level decreases and whether it is gradual or perhaps more abrupt. Similar situation with Tub1 overexpression in fig. 4B.

Rapid exchange and/or degradation of alpha tubulin:

6) The manuscript states, "We propose a model where cells use isotypes to create an excess of α-tubulin expression, and then rapidly exchange α-tubulin protein to ensure sufficient heterodimer production and prevent the accumulation of β-tubulin monomers." It is unclear how the data support a rapid exchange of alpha tubulin subunits. Can the data discriminate between a rapid exchange versus just partnering with newly made beta over several cell doublings? It seems like during the production of several fold more tubulin, simple competition for newly made beta could result in the overexpressed subunit becoming a larger percentage in tubulin heterodimers, even without any exchange. The tubulin assemblies slowly decrease over several hours, yet this could involve exchange or simply degradation of material in the assemblies. The data supporting rapid exchange over competition in synthesis needs to be more clearly described.

Lowering levels of an alpha isotype:

7) The manuscript states, "In contrast, when α-tubulin is overexpressed, levels of other α-tubulin isotypes decrease in response and ultimately return cells to endogenous levels of α-tubulin." This statement implies that the other alpha isotype is selectively decreased, when in actuality the levels of all alpha tubulins decrease. It's not a decrease of the other isotype that returns the cell to original endogenous levels. The overexpressed alpha also decreases. This overall decrease would require regulation at some level (expression, translation, degradation) which has not been yet shown. Significantly, the statement implies a selective downregulation of the other alpha, which the data do not support.

Model of specific isotypes downregulated:

8) In several other places the manuscript makes statements about the levels of certain alpha isotypes selectively decreasing. Some examples are:

"Conversely, increased α-tubulin is tolerated through an equilibration mechanism that decreases levels of an alternative α-tubulin isotype to return to a predetermined level of total α-tubulin."

"When protein levels of a new α-tubulin isotype increase, the other α-tubulin isotype is gradually lost from the cell until total α-tubulin returns to a predetermined set point."

These statements convey that when an alpha tubulin isotype is overexpressed, other alphas are selectively decreased. The data show that overall alpha tubulin is reduced, regardless of isotype, i.e. it's not the 'alternative' that is reduced because the overexpressed isotype is also reduced. In fact, the induced subunit even appears decreased from its high point to a larger extent, thus they may be lost proportionally to their levels. While this may be what the authors meant to convey, the statements above are vague and imply selectively when the data indicate a general decrease of alphas.

Other comments:

9) For Bim1 and Stu2 localization with Tub1 overexpression, the selected examples have tubulin assemblies that are quite difficult to make out. Could examples be shown that have prominent assemblies such as those shown for Bik1 and Kip3?

10) The amounts stated in the methods for purified protein loading are inconsistent with the amounts on westerns/graphs shown.

11) It's unclear whether the number of cells loaded on western blots was derived from the hemocytometer counts or the fraction of cells/colonies recovered (in Fig S2A). This would help readers interpret the data in Fig 2G.

12) The abstract states: "We provide evidence that cells equilibrate α-tubulin protein concentration during shifts in α-tubulin isotype expression to maintain a 20% excess of α-tubulin protein relative to β-tubulin protein" This statement should be more specific. While the data show that this is the case when alpha tubulin expression is shifted up, the data show this is not the case

when alpha tubulin expression is shifted down.

13) This sentence in the discussion seems unclear: "However, loss of α- or β-tubulin genes has little effect on the amount of corresponding tubulin detected in the cell (Figure 2)." Is this saying that the loss of a gene does not affect the protein levels of that same subunit? It seems as though it does (Fig 2G-H). Does "corresponding tubulin" in this sentence refer to the complementary (or other) subunit type being unchanged?

14) "Removing a single copy of TUB1 creates an 36% increase in β-tubulin in the cell (Figure 2GH)." Does the level of beta tubulin actually increase? Would it be better to say "excess" rather than "increase"?

Reviewer #2 (Comments to the Authors (Required)):

Overall, I think that the authors have responded well to the reviewer critiques by providing significant new data to address reviewer concerns and new text to go with these experiments, and they have also made the other suggested edits. In my opinion, the manuscript will be ready for publication after a few minor issues are addressed, all of which accomplished at the level of writing.

Minor concerns:
1) I am a bit puzzled that the authors did not respond to Review 1's major concern that "It has been previously and well-established that budding yeast TUB1 (alpha) and TUB2 (beta) is essential, and Tub3 (alpha) in non-essential. The current work, while introducing the term "tubulin assemblies" to describe aggregates of non-functional tubulins, and establishing concentrations of alpha and beta tubulins in budding yeast, does not shed further light on known observations."

In my opinion, this paper provides considerably cell biological, biochemical, and mechanistic depth to this long-established but puzzling observation. To avoid having other readers come to a conclusion similar to that of Reviewer 1, the authors could consider adding to the Discussion a more explicit statement about how their work relates to and extends beyond this earlier work.

We agree with this suggestion and have added the following sentences to the final paragraph of the discussion:

"It is well-established that TUB1 is an essential gene in haploid yeast, while TUB3 is non-essential (Schatz, Solomon, and Botstein 1986), and our data suggest a stronger requirement for TUB1 over TUB3 in diploid cells (Figure 1). This may be attributable to higher levels of α-tubulin expression from TUB1, compared to TUB3, which has been demonstrated by previous studies but recently called into question(Kilmartin 1981; Barnes, Louie, and Botstein 1992; Bode et al. 2003; Gartz Hanson et al. 2016; Nsamba et al. 2021). Nevertheless, our results suggest that maintaining two α-tubulin isotypes could provide a fitness advantage by together producing higher levels of α-tubulin."

2) The following sentence on page 15 is an improvement relative to the original, but still needs some edits: "Undersupplying tubulin by decreasing α- or β-tubulin gene copy number affects the length and polymerization rate of microtubule polymers, consistent with the expected equilibrium between soluble tubulin and microtubules." The balance between soluble and polymerized tubulin is not technically an equilibrium but a steady-state. Replacements for "equilibrium" might include "balance," "steady-state," or "relationship."

Also, because people outside the microtubule field might be confused by this point, it could be helpful to provide a reference.

We have made this change, replacing “equilibrium” with “balance” on page 15 and added a citation to a paper from the Goodson lab (PMID 31577530).

Reviewer #3 (Comments to the Authors (Required)):

This manuscript from Wethekam et al. takes a genetic based approach, controlling tubulin gene number in yeast, to address the central question of how overall tubulin heterodimer level, and relative level of alpha and beta tubulin subunits are controlled in vivo. The experiments are well planned and executed. While the revised manuscript does a good job of addressing most of the reviewer comments, before publication there remain some aspects of data presentation and interpretation that should be clarified. There are also instances of interpretations/conclusions that are not supported by the data presented and should be modified and/or more precisely stated.

Major comments:

Tubulin levels:

1) The authors have applied quality control approaches to the western blot data by removing data points that show decreased tubulin levels despite increasing lysate loading or overall linear regression with $r2<0.75$. This resulted in removal of some of the noisier experiments and increased linearity of the remaining points. The linearity of signal intensity is now presented in supplemental graphs. These data are then used to calculate the number of tubulin molecules per cell. As with the purified tubulin standards, the cell lysates would be expected to show a tubulin signal response that is proportional to the dilution.

Although the quality control methods increase linearity, they don't address the issue of proportionality of increase between tubulin signal/molecules and the amount of lysate/number of cells loaded. It appears that in most of the standard curves the tubulin intensity signals approximately triple from 5 to 15 ng loading. In many of the samples the tubulin signal appears to increase only a fraction (~30%) while cell number more than doubles. This gives the impression that the number of tubulin molecules calculated per cell would be far from linear across these dilutions. Rather than raw signal intensity, it would be more informative if the corresponding number of tubulins (calculated from the standard curve) is graphed against the lysate amount/number of cells.

Because these data are used to generate molecules per cell, rather than showing linear raw signal intensity, it would be much more informative if the corresponding number of tubulin molecules (calculated from the standard curve) per cell is graphed over the increase in number of cells loaded. If tubulin/cell is consistent across cell loading it would significantly strengthen the presentation. If not, it raises the question whether combining four values that may differ by 2-fold across the dilution series generates an accurate number of molecules per cell. If tubulin signal is not increasing proportionally with protein loaded, would it perhaps be more accurate to use the values calculated from the least loading? In either case it will help the reader to interpret the underlying data and resulting conclusions.

We thank the reviewer for raising this important point. We have now re-analyzed our data to address this concern. The key change in our revised analysis is that we use the Zwf1 loading control to estimate the number of cells represented by each lane, rather than assuming that the pipetted volumes in each replicate are consistent and accurate. This revised method is fully described in the materials and methods. To briefly summarize, we first used the Zwf1 loading control to estimate the number of cells loaded in each lane. For each replicate western blot, the integrated signal intensity for each band of the Zwf1 loading control was plotted as a function of estimated cells in the sample volume. The r^2 value for that relationship was calculated, and we used a cut off value of 0.8. For the few with lower r^2 values, we identified the outlier lane and excluded it. Next, the proportionality of Zwf1 signal was determined by dividing the measured Zwf1 signal intensity in each lane by the number of cells loaded in the lane. The average of those quotients is the expected Zwf1 signal per cell for that blot. We used that value to then recalculate the number of cells in each lane by dividing the Zwf1 signal intensity for each lane by the average Zwf1 signal per cell. We then used these adjusted cells/lane values to re-calculate the tubulin molecules per cell. This new calculation creates estimated tubulin/cell values that are more consistent across lanes, as evidenced by smaller standard deviations, and exhibit a stronger proportionality of tubulin signal as a function of cell number, which is now shown in revised supplemental figures 2 and 3. Importantly, this new analysis creates estimates of tubulin molecules per cell that are more consistent across biological and technical replicates for a given genotype, which is reflected in the revised panels 2E and 2F. This strengthens our conclusions about how gene copy number impacts tubulin protein levels.

Decreasing tubulin levels over 24 hours:

2) The overexpressed alpha isotype decreases from 3 to 24 hours as much or more than the non-overexpressed alpha. This seems to show the decrease in alpha is a general response on overall alpha level. As the authors state, this could occur at multiple points in tubulin proteostasis. Alternatively, it's unclear if the cells/cultures are still in exponential phase at the 24-hour timepoint. If not, might this affect expression levels? If the cells/cultures were kept in exponential phase, can it be excluded that there is any downregulation of GAL

expression over 24 hours, or that selective pressure enriched for cells that express less tubulin, e.g., the cells overexpressing alpha tubulin doubled only once in the 6-hour videos.

We now include a new experiment that addresses this point by measuring the rate of tubulin loss after shutting off transcription from the GAL promoter. In this experiment, we induce GFP-Tub3 with galactose for 2 hours, shut off transcription by adding glucose, and then measure alpha protein levels at one-hour intervals for 5 hours after shutoff. These data are now included as Figure 5H-J and show that excess α-tubulin is degraded within a few hours of transcriptional shut off. Importantly, we also include a similar experiment for beta tubulin (Figure 5N-P) and find a different result – beta levels persist for hours after shut off. These new results strengthen our conclusion that excess alpha tubulin is readily degraded while excess beta tubulin persists for hours after its biogenesis.

3) The discussion states: "The autoregulation model predicts that this increase in soluble tubulin should induce mRNA decay and thereby inhibit further translation, creating a steady state level of β-tubulin. In our experiments, β-tubulin levels continue to increase for hours without reaching steady state levels (Figure 3E-M) [this may be an incorrect callout to Fig 5H-J?]. In contrast to α-tubulin, we speculate that super-stoichiometric β-tubulin may form 'dead-end' assemblies that exhibit very slow exchange and are not readily targeted for degradation (Figure 6)." This statement overstates the data on beta tubulin levels and seemingly draws an unsupported conclusion between alpha and beta levels. The data in Fig 5I show beta levels are steady from 3-5 hours, and given the SD are likely statistically indistinguishable from 2-5 hours. Thus, they increased before 2 hours but remain at steady state until the end of the time course. Whether they remain there beyond 5 hours, increase at later time points, or perhaps decrease is not known.

As mentioned in our response to the previous point, we now include new results from shutoff experiments indicating that excess α-tubulin is rapidly degraded whereas excess β-tubulin persists. This supports our speculation regarding β-tubulin assemblies. We have now re-written these sentences in the discussion on page 16 to read:

"Our results indicate that budding yeast regulate β-tubulin differently than α-tubulin. β-tubulin does not appear to access an alternative state that can be rapidly turned over. When we induce TUB2 overexpression, the levels of the native β-tubulin are not obviously affected by the increase of the induced β-tubulin (Figure 5M) . Furthermore, when we shut off TUB2 overexpression, β-tubulin levels remain elevated for hours, which is in contrast to the rapid loss of excess α-tubulin after shutting off its transcription. We speculate that the persistence of excess β-tubulin may reflect the formation of 'dead-end' β-tubulin assemblies that exhibit slow exchange and are not readily targeted for degradation (Figure 6)."

We have also corrected the call out as suggested.

4) Near the end of results on page 14 states: "This suggests that β-tubulin expression does not equilibrate like α-tubulin expression." The total levels of alpha (5F, as well as 4B) and beta (5I) tubulin following induction look similar at 0, 2, and 3 hours. But this is where the comparison between alpha and beta ends. Beta tubulin average is slightly increased (but essentially steady state given SD) at 4 and 5 hours, but alpha tubulin is not examined in this range. Alpha tubulin is only examined at 24 hours, but beta tubulin (for technical reasons) is not examined beyond 5 hours. Unless the authors can explain something more clearly, the only difference that can be gleaned from this comparison is the decrease in Tub1p at 3 hours (Fig 5G) compared to the steady level of Tub2-438D over the same period (Fig 5J), i.e., there is no indication whether beta levels would equilibrate back to near normal levels or not.

We agree that this conclusion cannot be drawn from comparing the levels of α- vs β-tubulin after 2 and 3 hours of induction. This question motivated us to perform the new shut off experiments that more closely examine α- and β-tubulin levels after shutting off induction, and using the same time course. The new results in Figure 5H-J and N-P more clearly demonstrate that excess α-tubulin is rapidly lost after shutting off its transcription, but β-tubulin persists for the 5-hour time course of our experiment.

We have re-written this concluding sentence to read:

"This suggests that excess β-tubulin is not rapidly turned over like excess α-tubulin."

5) The manuscript states: "Our western blots show that total α-tubulin is increased approximately two-fold at 2 hours post induction, followed by a gradual decrease that returns to preinduction levels by 24 hours (Figure 5E, F)." The 3-hour point is equal or higher than the 2-hour level, and the only next point examined is at 24 hours. There is no data showing when the level decreases and whether it is gradual or perhaps more abrupt. Similar situation with Tub1 overexpression in fig. 4B.

We have removed the word "gradual" from this sentence on page 12. Our new experiments measuring tubulin loss after transcriptional shut off (Figure 5H-J, N-P) also address this concern by providing evidence of tubulin loss with higher time resolution.

Rapid exchange and/or degradation of alpha tubulin:

6) The manuscript states, "We propose a model where cells use isotypes to create an excess of α-tubulin expression, and then rapidly exchange α-tubulin protein to ensure sufficient heterodimer production and prevent the accumulation of β-tubulin monomers." It is unclear

how the data support a rapid exchange of alpha tubulin subunits. Can the data discriminate between a rapid exchange versus just partnering with newly made beta over several cell doublings? It seems like during the production of several fold more tubulin, simple competition for newly made beta could result in the overexpressed subunit becoming a larger percentage in tubulin heterodimers, even without any exchange. The tubulin assemblies slowly decrease over several hours, yet this could involve exchange or simply degradation of material in the assemblies. The data supporting rapid exchange over competition in synthesis needs to be more clearly described.

Our microscopy analysis in Figure 5 and Videos 1-3 indicate that newly expressed GFP-Tub3 forms heterodimers and incorporates into microtubules quickly (<1hr) and does not require rounds of cell division. We interpret this as newly expressed α-tubulin incorporating into the heterodimer pool by partnering with both pre-existing β-tubulin (i.e., rapid exchange) and by partnering with newly synthesized β-tubulin (competition in synthesis). These models are not mutually exclusive, but the key distinction is the rate of incorporation into the heterodimer pool.

If α-tubulin could only use competition in synthesis, then incorporation would be limited by the rate of β-tubulin biogenesis. Tub2/β-tubulin biogenesis appears to be slow. Our results and previous estimates of half-life indicate that Tub2/β-tubulin is a very long-lived protein. Analysis by the Walther lab estimates the half life of Tub2/β-tubulin to be ~6 hours and primarily driven by titration due to cell division (PMID: 25466257). This is consistent with our shut off experiments in Figure 5N-P, which show that when we transiently express Tub2 its protein level remains constant for longer than 5 hours after induction. If Tub2 protein is stable across multiple rounds of cell division, then the normal rate of biogenesis would supply half of the cell's Tub2 pool per division. Based on our measurements of doubling time and β-tubulin molecules per cell, the rate of biogenesis needed to replenish half of cell's β-tubulin would be approximately 3750 molecules per hour. We estimate that the normal rate of normal α-tubulin biogenesis is faster, at least 5750 molecules per hour. Galactose-induced α-tubulin would need to be 3X faster to produce a 70% excess of α-tubulin during the first hour of induction. Therefore, during that first hour of GFP-Tub3 induction in our Figure 5 experiment, new α-tubulin would outnumber new β-tubulin 6:1, and only a small fraction of GFP-Tub3 protein could partner with β-tubulin to form heterodimers. This model of limited β-tubulin availability is not sufficient to explain our observations of the rapid appearance of GFP-Tub3 in microtubules, followed by its accumulation in the cytoplasm and in assemblies

On the other hand, if α-tubulin could rapidly exchange then newly expressed GFP-Tub3 could partner with both pre-existing and new β-tubulin. In this case, the total ratio of α:β after one hour of induction is ~3:1, and a larger fraction of GFP-Tub3 could partner with β-tubulin to form heterodimers. This appears to be more in line with our observations.

Lowering levels of an alpha isotype:

7) The manuscript states, "In contrast, when α-tubulin is overexpressed, levels of other α-tubulin isotypes decrease in response and ultimately return cells to endogenous levels of α-tubulin." This statement implies that the other alpha isotype is selectively decreased, when in actuality the levels of all alpha tubulins decrease. It's not a decrease of the other isotype that returns the cell to original endogenous levels. The overexpressed alpha also decreases. This overall decrease would require regulation at some level (expression, translation, degradation) which has not been yet shown. Significantly, the statement implies a selective downregulation of the other alpha, which the data do not support.

We agree with the reviewer that our data do not support selective decrease of the other isotype. To avoid this impression, we have now re-written this sentence on page 12 as follows:

"Together, these data suggest that overexpression transiently increases levels of α-tubulin and stimulates rapid protein turnover that includes depletion of other α-tubulin isotypes to return to pre-induction levels of total α-tubulin."

Furthermore, our new shut off experiments (Figure 5H-J and N-O) indicate that degradation of excess α-tubulin is likely play an important role in return total α-tubulin to pre-induction levels.

Model of specific isotypes downregulated:

8) In several other places the manuscript makes statements about the levels of certain alpha isotypes selectively decreasing. Some examples are:

"Conversely, increased α-tubulin is tolerated through an equilibration mechanism that decreases levels of an alternative α-tubulin isotype to return to a predetermined level of total α-tubulin."

"When protein levels of a new α-tubulin isotype increase, the other α-tubulin isotype is gradually lost from the cell until total α-tubulin returns to a predetermined set point."

These statements convey that when an alpha tubulin isotype is overexpressed, other alphas are selectively decreased. The data show that overall alpha tubulin is reduced, regardless of isotype, i.e. it's not the 'alternative' that is reduced because the overexpressed isotype is also reduced. In fact, the induced subunit even appears decreased from its high point to a larger extent, thus they may be lost proportionally to their levels. While this may be what the

authors meant to convey, the statements above are vague and imply selectively when the data indicate a general decrease of alphas.

We agree with the reviewer that our results do not clearly demonstrate selective decrease of a particular isotype when another isotype is overexpression. To avoid giving the reader that impression, we have re-written the two sentences cited by the reviewer as follows:

Page 14:
"Conversely, increased α-tubulin is tolerated through an equilibration mechanism that decreases excess α-tubulin to return to the predetermined level of total α-tubulin."

Page 13:
"Together these data suggest that increased levels of α-tubulin protein stimulates the turnover of the α-tubulin pool in the cell."

Other comments:

9) For Bim1 and Stu2 localization with Tub1 overexpression, the selected examples have tubulin assemblies that are quite difficult to make out. Could examples be shown that have prominent assemblies such as those shown for Bik1 and Kip3?

We have now replaced these images in Figure 4G with different examples that more clearly show tubulin assemblies.

10) The amounts stated in the methods for purified protein loading are inconsistent with the amounts on westerns/graphs shown.

We now clarify this point in the Materials and Methods (page 25):

"Samples containing increasing amounts of cells (3.5, 4.5, 6, and 8 x 10^6) or purified tubulin (4,10, 15, 30 and 40 ng of total protein heterodimers or 2, 5, 7.5, 15, and 20 ng of each α- or β-tubulin subunit) were loaded and blotted as described above."

11) It's unclear whether the number of cells loaded on western blots was derived from the hemocytometer counts or the fraction of cells/colonies recovered (in Fig S2A). This would help readers interpret the data in Fig 2G.

We now clarify this point in the Materials and Methods (page 25):

"To prepare lysate of 5x10^7 cells in 50 µL samples, cells were counted on a hemocytometer and the appropriate volume of cells was determined based on the hemocytometer counts and prepared as described above."

12) The abstract states: "We provide evidence that cells equilibrate α-tubulin protein concentration during shifts in α-tubulin isotype expression to maintain a 20% excess of α-tubulin protein relative to β-tubulin protein" This statement should be more specific. While the data show that this is the case when alpha tubulin expression is shifted up, the data show this is not the case when alpha tubulin expression is shifted down.

We have now re-written this sentence for clarity:

"We provide evidence that yeast cells equilibrate α-tubulin protein concentration when α-tubulin isotype expression is increased and normally maintain an excess of α-tubulin protein relative to β-tubulin protein."

13) This sentence in the discussion seems unclear: "However, loss of α- or β-tubulin genes has little effect on the amount of corresponding tubulin detected in the cell (Figure 2)." Is this saying that the loss of a gene does not affect the protein levels of that same subunit? It seems as though it does (Fig 2G-H). Does "corresponding tubulin" in this sentence refer to the complementary (or other) subunit type being unchanged?

We have now re-written this sentence on page 14 for clarity:

"Loss of one copy of the major α-tubulin gene TUB1 leads to a slight decrease in the amount of α-tubulin protein in the cell, accompanied by a strong increase in the amount of β-tubulin (Figure 2G). In contrast, loss of one copy of the β-tubulin gene TUB2 decreases β-tubulin protein levels but does not impact α-tubulin levels (Figure 2G)."

14) "Removing a single copy of TUB1 creates an 36% increase in β-tubulin in the cell (Figure 2GH)." Does the level of beta tubulin actually increase? Would it be better to say "excess" rather than "increase"?

Our analysis shows that the level of β-tubulin is significantly higher when one copy of TUB1 is ablated, compared to wild-type cells in a pair-wise Student's t test. Therefore it is accurate to state that β-tubulin is increased, and not simply in excess of α-tubulin.

October 3, 2022

RE: JCB Manuscript #202202102RR

Dr. Jeffrey K Moore
University of Colorado Anschutz Medical Campus
12801 E 17th Ave
MS8108
Aurora, Colorado 80045

Dear Dr. Moore,

Thank you for submitting your revised manuscript entitled "Asymmetric requirement for α-tubulin over β-tubulin." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add MW markers to Figures 2E/F 3B, 4A, 5E/H/K/N/Q, & S5H.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Title: We feel that the current title does not fully communicate the main theme of the work and therefore suggest the following title: "Tubulin isotype level regulation sustains an asymmetric requirement for α-tubulin over β-tubulin"

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial please add a reference citation if possible.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes

f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item.

11) Video legends: Should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments, concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

12) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

13) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

14) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

15) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

16) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

Please provide Source Data for Figures 4A & S5H.

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B. FINAL FILES:

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-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

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