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More than two populations of microtubules comprise the dynamic mitotic spindle

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The decay kinetics of photomarked tubulin is frequently used to calculate the turnover of various microtubule populations in the mitotic spindle. Curiously, decay kinetics are best fit by a bi-exponential function, and this has been taken by the field to reflect kinetochore- and non-kinetochore microtubules. This is very likely to be a dated idea. We now know that the architecture of the mitotic spindle is significantly more complex than previously thought; for example, microtubules are nucleated throughout the spindle (via augmin), bridging fibers connect sister kinetochore-fibers, and interpolar microtubules link the two spindle halves together.
This study calls into question the meaning of the bi-exponential decay kinetics exhibited by the mitotic spindle. Microtubule turnover rates are measured after different perturbations are made, e.g., depletion of proteins required for end-on kinetochore microtubule attachments, inhibition of a motor (Eg5) required for spindle bipolarity, and after inhibition of a kinase (Aurora B) that regulates kinetochore microtubule attachments. In all cases, the authors find that microtubule dynamics are best described by bi-exponential kinetics although the rate constants and relative sizes of the two populations change. The authors conclude that current views regarding the nature of microtubule turnover data in the spindle are too simplistic, and that care must be taken in interpreting the meaning of such data.

Comments for the author

Because I feel that this will be helpful for the review process, I will state that I reviewed a form of this manuscript when it was submitted to Molecular Biology of the Cell. I was in favor of the work being published then, and my opinion has not changed. In the interim time, the authors have added additional experiments to the paper (e.g., measuring microtubule turnover in monopolar spindles), and this elevates my enthusiasm for the work. The most tricky aspect about this study is that it is challenging to interpret experiments in cells which contain k-fibers versus those that do not. For example, non-K-fiber microtubules may organize differently in cells with k-fibers versus cells lacking k-fibers. It is therefore possible that a direct comparison of normal cells to Hec1-depleted cells is not appropriate. I am glad to see that the authors have addressed this possibility in the Discussion. Collectively, I strongly believe that it is important for the field to read and evaluate this study, because it will cause other investigators to more carefully consider the meaning of their data. The spindle is a complex macromolecular structure, and we know much more of its architecture than we did in the 1990s. How we interpret data using older techniques must be more rigorously considered (or at least discussed).

Reviewer 2

Advance summary and potential significance to field

In the present manuscript, Tipton and Gorbsky revisit the use of fluorescence dissipation after photoactivation (FDAPA) of tubulin to infer about the different spindle microtubule populations in human cells. Classic work where this approach was pioneered (Zhai et al., 1995) convincingly demonstrated that more than one population of microtubules exist in the spindle, in agreement with several extensive electron microscopy analyses. More precisely, the fluorescence dissipations curves were best fit by a double exponential, suggesting the existence of two major microtubule sub-populations, with faster and slower turnover. Also based on our knowledge of the different stability of spindle microtubule sub-populations (e.g. cold resistance of kinetochore microtubules), the populations with faster and slower turnover were interpreted as representing non-kinetochore and kinetochore microtubules, respectively. The present work challenges this view after exploring many different experimental scenarios that should more specifically affect either of these two microtubule sub-populations.
Of particular relevance, the authors performed FDAPA of tubulin after depleting Hec1 by RNAi, which presumably prevents the formation of stable end-on microtubule attachments at kinetochore. If this assumption is correct, then the slower turnover population of spindle microtubules should disappear. However this was not the case and the authors propose that other microtubule subpopulations exist in the spindle. Because a FDAPA approach is now considered the

gold standard to investigate non-kinetochore and kinetochore microtubule dynamics, this work is important in its effort to clarify exactly what is being measured in such assays. It is therefore of interest to a wide audience of mitosis, kinetochore and spindle researchers. The authors are in general very careful with their claims and openly recognize potential caveats in the data interpretation. Yet, before claiming the main conclusion of this work, several alternative interpretations must be taken into consideration and additional controls/quantifications provided before I can recommend publication of this work in Journal of Cell Science. A detailed critique follows below.

Comments for the author

Major issues:

1- The authors readily recognize in their discussion that "new" microtubule populations may arise due to the induced perturbations and this is to be applauded. Nevertheless, alternative interpretations should also be considered.
For instance, the authors compare Hec1 depleted cells (+MG132) in which most chromosomes are misaligned, with control metaphase cells (+MG132). Wouldn't it be more reasonable/fair to draw comparisons between Hec1 depleted cells and control early PROMETAPHASE cells? The early prometaphase state might actually correspond well to what is found after Hec1 depletion (see e.g. Steblyanko et al., EMBO J 2020), where mostly lateral interactions between kinetochores and spindle microtubules predominate. Based on a quick literature survey (mostly from extensive work by the Compton Lab, but there are others), the slower turnover population of MTs in prometaphase is about 2x less stable compared to metaphase, which is roughly what the authors find for Hec1-depleted cells.

Likewise, the STLC condition used in the present manuscript, also mimics the prometaphase condition where the stability of end-on kinetochore-microtubule attachments in this case is known to be compromised due to the lack of tension (see the Mad2 data in Kapoor et al., JCB 2000). Altogether, the Hec1 RNAi (and STLC) data, seminal for the main conclusion of this paper, may not highlight a different stable microtubule population in the spindle, but rather reflect the dynamics of less mature/incomplete kinetochore-microtubule attachments (or even just a more stable microtubule population) typical of early prometaphase cells.

This is a critical point that must be clarified experimentally. I also wondered whether the reason why the authors could not obtain good regression curve fits after inhibiting Eg5 and partially depleting Hec1 is precisely because now the slower turnover population is nearly absent (see next point)

2- Related to the previous point, it is still visible from the time lapse images with SiR-DNA, that Hec1-depleted cells still have a significant fraction of chromosomes that remain tethered to the spindle and align at the equator.
This is not surprising, since end-on attachments are not required for chromosome alignment (Cai et al., NCB, 2009), which can be mediated by CENP-E-dependent sliding along pre-existing microtubules (Kapoor et al., Science, 2006). Because CENP-E is also involved in the lateral-to-end-on conversion of kinetochore-microtubule attachments (Shrestha and Draviam, Curr Biol, 2013), it remains possible that transient end-on attachments still take place in the Hec1-depleted conditions used in the present manuscript. Indeed, few cold-stable MT bundles with lateral-associated kinetochores have been reported after Ndc80 knockdown in different systems (DeLuca et al., MBoC, 2005; Feijao et al., Cytoskeleton 2013). Therefore, it is possible that the detectable slower turnover population after Hec1 RNAi simply represents residual/transient kinetochore microtubule bundles. The authors should use immunofluorescence analysis in fixed cells to investigate the presence of cold-resistant (5-10 min) microtubule bundles and their attachment status to kinetochores after their Hec1 RNAi conditions. They may also eventually want to compare this with the Eg5 inhibition and partial Hec1 depletion.

3- One interesting observation from the present study is the increased stability of the slower turnover microtubule population after prolonged MG132 treatment. Since Hec1 depletion prevents SAC satisfaction (yet there is also the issue of Hec1 depletion compromising SAC response…), I assume that the imaged cells have been in mitosis for a while and wondered whether any eventual delay associated with Hec1 depletion could also account for the appearance of a slower turnover

microtubule population (related to the previous point). Could the authors indicate what fraction of Hec1-depleted cells under their experimental conditions is SAC proficient or deficient and discuss the results relative to the point being raised?

4- The authors use a combination of Hec1 depletion and Aurora B inhibition to conclude that Aurora B stabilizes a non-kinetochore microtubule pool with a slower turnover. However, the differences relative to Hec1 inhibition alone are borderline, if significant at all (3.3+/- 0.3 Hec1 RNAi vs 2.2 +/- 0.3 min Hec1 RNAi+ZM). My reading of these numbers is that there is little or no effect on the slower turnover microtubule population of Aurora B inhibition under conditions in which end-on attachments are already compromised by Hec1 depletion. Indeed, Aurora B inhibition alone strongly stabilized the slower microtubule population, but it would not be expected to do so if this population is already compromised. This requires some discussion and eventually re-interpretation of the data.

Minor issues:
1- The low temperature condition is interesting but not very informative.
The observed defects might simply reflect a global slow-down of all metabolic processes in cells that indirectly impact microtubule dynamics. Please discuss.
Helder Maiato

First revision

Author response to reviewers' comments

Reviewer 1 We thank the reviewer for the strong comments concerning the importance of our work and the impact it will have on the field of spindle structure and function. The reviewer did not request additional experiments or changes to the text.

Reviewer 2, self identified as Dr. Helder Maiato We thank Dr. Maiato for his careful consideration of our work and for his statement "Because a FDAPA approach is now considered the gold standard to investigate non-kinetochore and kinetochore microtubule dynamics, this work is important in its effort to clarify exactly what is being measured in such assays. It is therefore of interest to a wide audience of mitosis, kinetochore and spindle researchers."

Major issues:
Issue 1 – Dr Maiato makes several salient points in this paragraph, which we have addressed experimentally. He points out the potential effects of immature or residual end-on attachments in Hec1-depleted cells and in cells treated with Eg5 inhibitors that would cause such cells to resemble prometaphase cells in their microtubule attachments. To address this issue we carried out extensive fluorescence dissipation assays in prometaphase U2OS cells. As has been reported by others, notably the Compton laboratory, we detected an overall increase in stability of the slow population in metaphase versus prometaphase cells though the difference was not as large as the difference between metaphase cells and Hec1-depleted cells presented originally (see revised Fig 1). Moreover while the trend toward increased stability was present comparing prometaphase and metaphase cells, it did not rise to the level of traditional statistical significance. We note this trend follows the reports from the Compton lab for U2OS cells (Bakhoum et al. Nature Cell Biology volume 11, pages 27–35, 2009) and for RPE-1 cells (Kabeche and Compton, Current Biology, volume 22, pages 638-644, 2012). However in neither case did the studies report statistical analysis comparing prometaphase and metaphase cells. We included the speculation that prometaphase cells likely contain a mix of kinetochore attached and transiently anchored microtubules (page 4, lines 17-20). However, given the more extreme changes in slow population turnover we detected in Hec1-depleted cells, we conclude that such cells are likely to reflect an even greater loss of kinetochore attachment that could be detected in prometaphase.

We also point out several technical issues that may compromise direct comparison of our studies with those reported previously. First we found highly significant increases in slow population stability in cells incubated for long periods in proteasome inhibitor. Therefore we only used data

for cells that we saw enter mitosis in the course of the experiment. Kabeche and Compton, 2012 reported that proteasome inhibitor did not affect metaphase microtubule turnover. While this difference may be a cell type specific variation, we believe imaging metaphase cells after long incubation in proteasome inhibitor could account for some discrepancies. Second, we had the advantage of using cells expressing mCherry tubulin along with the photoactivatable GFP, whereas most previous studies used cells only expressing photoactivatable GFP. This allowed us to assure that spindles were not rotating out of the plane of focus, a particular problem in prometaphase where spindles are more mobile. Such rotation would lead to a loss of fluorescence that might be incorrectly interpreted as fluorescence dissipation.

Issue 2 Dr Maiato raises the issue of potential lateral interactions of chromosomes via kinetochore proteins such as CenpE (and we would add kinetochore-associated dynein, see our paper Vorozhko et al., Chromosoma, volume 117, pages 169-79, 2008). He asserts that our images of DNA in Hec1-depleted cells "have a significant fraction of chromosomes that remain tethered to the spindle and align at the equator." While we agree that chromosomes in Hec1 depleted cells remain tethered to the spindle (see Vorozhko et al., 2008, cited above), we disagree that the chromosomes in these cells align at the spindle equator to any significant degree. The images referred to are shown in Fig S2. There, chromosomes remain associated with the spindle but are spread across the whole spindle and move about the spindle randomly, only transiently moving near the spindle equator. Contrast the distribution of chromosomes in this example with that of cells treated with inhibitor to CenpE where a majority of chromosomes align at the metaphase plate while a minority remain near the poles (see for example Barisic et al., Nature Cell Biology, 16, pages 1249–1256, 2014). Dr. Maiato then suggests carrying out a large number of experiments treating cells with cold to characterize cold-resistant microtubules. We elected to not do this extensive set of experiments for several reasons. First they would be redundant with studies previously carried out by several laboratories including the references highlighted in the review. Most importantly, in our hands cold treatment experiments generate highly variable results in a field of cells, even with controls. When cells are treated with cold the spindles immediately shrink and the spindle poles collapse onto the chromosomes. We question whether the stable fibers retain reflect a significant portion of the original kinetochore fibers. Most importantly, in examining cells with apparently aligned metaphase chromosomes, one finds a wide range of residual microtubule morphologies. While a few cells show spindle-like remnants, the vast majority of cells exhibit very short bundles that associate with some of the chromosomes and bear little resemblance to mitotic spindles. Note the examples provided in the recent, comprehensive analysis of this technique from the Compton lab (see Fig 3C and 3D in Warren et al., Methods in Cell Biology 158, pages 91–116, 2020). The authors in that study concluded that cold treatment was unable to detect differences in microtubule dynamics comparing prometaphase versus metaphase cells nor differences in dynamics for control cells and cells treated with a microtubule disruptor. Because of the very limited likelihood of obtaining novel, useful data plus limited laboratory resources due to the pandemic, we concluded that carrying out cold treatment experiments would not enhance conclusions obtained in our already extensive analysis.

Issue 3 First, in the middle of this paragraph Dr Maiato states "I assume that the imaged cells have been in mitosis for a while and wondered whether any eventual delay associated with Hec1 depletion could also account for the appearance of a slower turnover microtubule population (related to the previous point) signaling." As noted in the original manuscript, we only used cells that we saw enter mitosis during the course of the experiment to eliminate the complication of cells changing their dynamics during an extended time in M phase. This technical issue was included in the original manuscript and is emphasized in the revised version (page 3, lines 19-21 and in methods, page 9, lines 36-37). In short, cells we analyzed had not, in fact, been in mitosis for an extended period and therefore in our experiments the appearance of a slower turnover microtubule population could not be attributed to mitotic delays induced by the Hec1 depletion. Dr. Maiato then requested we address the competency of our Hec1-depleted cells in signaling the spindle checkpoint (or SAC). Historically while our laboratory in collaboration with the Stukenberg laboratory showed that spindle checkpoint signaling was abrogated and M phase cells spontaneously exited mitosis upon inhibition of Hec1/Ndc80 through antibody microinjection experiments (McCleland et al., Genes and Development, 17, pages 101-14, 2003). However, contemporary siRNA studies suggested that the phenotype of Hec1/Ndc80 depletion was mitotic arrest (Martin-Lluemsa et al., Science, 297, pages 2267-70; DeLuca et al., Current Biology, 13, pages 2103-9, 2003). The Sorger lab (Meraldi et al., Developmental Cell, 7, 45-60, 2004) then

showed that a minority of cells treated with siRNA to Ndc80 complex components would override the spindle checkpoint and escape mitosis, and that this proportion could be increased by cell synchronization methods. These observations led to the development of the two stage phenotype of Hec1/Ndc80 depletion. Significant depletion would abrogate end-on kinetochore attachment but more robust inhibition was required to eliminate spindle checkpoint signaling. To test and quantify spindle checkpoint signaling in our Hec1 depletions, we measured Mad2 accumulation at kinetochores (Fig S4C). In cells with intact microtubules, Hec1 depletion significantly reduced Mad2 accumulation on kinetochores to levels comparable to that seen at control metaphase cells. In cells treated with the microtubule disruptor, nocodazole, Hec1 depletion again significantly reduced Mad2 accumulation at kinetochores.

Issue 4 We concur with Dr. Maiato's suggestion that comparison of the effects on slow turnover population microtubules, with and without Aurora B inhibition, in cells depleted of Hec1 would be useful and appropriate. As requested, this comparison with discussion has been added to the manuscript along with appropriate caveats regarding the interpretation of the results (page 7, lines 14-27).

Minor Issue 1 We have added the caveat about the potential indirect effect of lowered temperature on the metabolic activity of the cells in the temperature comparison experiments (page 8, lines 18-21)

Second decision letter

MS ID#: JOCES/2021/258745

MS TITLE: More than Two Populations of Microtubules Comprise the Dynamic Mitotic Spindle

AUTHORS: Aaron R Tipton and Gary J Gorbsky
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This is an important paper for researchers in the mitosis community, and the microtubule field more broadly to read. The study is from a scholar in the field of mitosis, and it it, the authors evaluate the long-held notion that the mitotic spindle is only comprised of two populations of microtubules: kinetochore-microtubules and non-kinetochore microtubules. This assumption came from early studies, most notably Zhai et al. (1995), which showed that microtubule turnover in the spindle exhibits bi-exponential kinetics. The authors of Zhai et al. interpreted this to mean that the slow turnover population corresponds to kinetochore-microtubules, and that the fast turnover population represents non-kinetochore microtubules. We now know that the spindle is more complex in its organization, and it is totally reasonable that the previous interpretation of microtubule turnover kinetics is overly simplistic. Work in this study is consistent with this notion; eliminating kinetochore-microtubules (via Hec1 depletion) still produces decay curves that are best fit with a bi-exponential function.

Therefore, future work can (and should) be directed at investigating the potentially coincidental connection between bi-exponential turnover kinetics and microtubule populations in the spindle. More importantly, it is essential for researchers to interpret their microtubule turnover data with more caution. In reading critiques by Dr. Maiato, I do feel that this reviewer raised reasonable points that the authors should address. The authors did a brilliant job of responding to criticisms, performing experiments as necessary. In addition, I will say that writing of the paper has improved

in the 3 times that I have reviewed it. I strongly advocate for publication of the manuscript in its current form. The core results will not change with additional revisions, and the field should be aware of this important work.

This reviewer is identifying himself as Ryoma Ohi (University of Michigan)

Comments for the author

None

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily addressed all my concerns. This mostly required recognition of potential caveats and experimental limitations, as well as including few additional controls to infer the extent of Hec1 depletion and comparison with control prometaphase cells. Overall, I think this paper is now acceptable for publication in Journal of Cell Science and will attract the interest of a broad readership of mitosis researchers. I congratulate the authors for this important contribution.

Helder Maiato

Comments for the author

no additional comments