Microtubule pivoting enables mitotic spindle assembly in S. cerevisiae

Kimberly Fong, Trisha Davis, and Charles Asbury

Corresponding Author(s): Charles Asbury, University of Washington School of Medicine and Trisha Davis, University of Washington School of Medicine

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(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.202007193

September 23, 2020

Re: JCB manuscript #202007193

Charles Asbury
University of Washington
Physiology & Biophysics
1959 NE Pacific St, HSB G-424
Seattle, Washington 98195-7290

Dear Dr. Asbury,

Thank you for submitting your manuscript entitled "The importance of being flexible: Microtubule pivoting enables mitotic spindle assembly". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers are quite positive regarding your study. We do find that the new experiments suggested by reviewer #2 seem straightforward and important, therefore please ensure they are thoroughly addressed, in addition to the requested text changes.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Reports may have up to 5 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.

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As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread

of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on 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.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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 in this letter.

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

Sincerely,

Melissa Gardner
Monitoring Editor

Andrea L. Marat
Senior Scientific Editor

Journal of Cell Biology

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

Assembly of bipolar mitotic spindles accompanies spindle pole separation. A prerequisite for this process is formation of pole-to-pole antiparallel microtubules that nucleate from each pole. Although our understanding of the molecular pathways leading to spindle assembly has been substantially improved during the last decade or so, details of the initial step that promotes antiparallel microtubule formation and molecules involved in this process are still largely elusive. Previous work performed in fission yeast showed that microtubule pivoting would be crucial for bipolar spindle formation, but how this is achieved at the molecular level is not fully understood. In this manuscript, Fong et al. have addressed this unsolved question in budding yeast, an organism that is supposed to need a strict regulatory system for this process.

The authors picked up Spc110, a component of the spindle pole body (SPB), for analysis of microtubule pivoting. Spc110 belongs to a widely conserved protein family, contains a long internal coiled-coil stretch and is known to function as a structural linker between the SPB and the microtubule minus-end-capping and nucleator complex (the gamma-tubulin ring complex). The idea behind this is that the long, internal coiled-coil stalk that contains several hinges/breaks might provide Spc110 with structural flexibility that promotes microtubule pivoting. Given this assumption, they first created four types of mutant strains that contain different Spc110 variants. CCΔ lacks two-thirds of the coiled-coil stalk (expected to be less flexible), GCN4 contains 33 amino acid residues derived from well-characterized, leucine zipper at the junction within CCΔ (expected to be stiff), SKIP4 contains a distinct, flexible hinge at the junction (expected to be flexible), and finally

TSMOD replaces the tension sensor module for a 124-residue portion of the coiled coil stalk (expected to be very flexible). Then, SPBs from individual strains were purified and in vitro assay of microtubule pivoting performed. Interestingly, and consistent with the proposition, individual SPBs displayed the varied degrees of microtubule pivoting with the order of TSMOD, SKIP4, wild type, GCN4 and CC□. Having obtained the in vitro data for individual Spc110 constructs, the authors next characterised in vivo behaviours of these strains, in particular focussing on the timing of SPB separation upon onset of mitosis. Largely in line with the data from in vitro assay, CC□ exhibited substantial delay in SPB separation, while TSMOD showed almost the same timing, and GCN4 and SKIP4 displayed modest delay. More detailed analysis performed in wild type and GCN4 cells showed that the timing of SPB separation was indeed delayed in GCN4 by 7 min compared to wild type cells.

This study contains fairly interesting new findings on microtubule pivoting and provides new insight into the initial stage of spindle assembly. In particular, in vitro assay is beautifully performed and well presented. I would like to raise a couple of points, and I hope that the authors could address them.

Major comments:
1) Basic characterisation of Spc110 mutant strains
Compared to the results from in vitro assay, those of microtubule pivoting in cells are less convincing. I understand the visualisation of microtubule pivoting would be technically challenging, but a few more data on Spc110 mutant proteins might be required. First, the authors seem to postulate that the amount of individual Spc110 mutant proteins is the same as that of wild type, but no data is presented. I think it is important to show this by simple immunoblotting or quantification of individual Spc110 proteins on SPBs. Second, it is important to show recruitment of the gamma-tubulin complex to the SPB is not compromised in each mutant.

2) Observation of spindle morphology
GCN4 cells display 7 min delay in the timing of SPB separation (Figure 4B). It would be nice to show live imaging of spindle morphology in parallel with wild type cells. Is there any difference in spindle intensity between GCN4 and wild type cells? If microtubule pivoting is defective in GCN4, spindle intensity during this delay period might be reduced.

Minor comments:
3) the title
The current title may not be the best choice, as it looks like a review article. I suggest a new title such as "Microtubule pivoting promotes mitotic spindle assembly in budding yeast"

4) Figure 1C, the legend
Please explain black arrowhead.

5) Figure 4, the legend
"cdc28-as1 cells (gold) versus wild type cells (blue)" should be "cdc28-as1 containing GCN4 cells (gold) versus cdc28-as1 containing wild type Spc110 cells (blue)".

6) Figure 4B
Image of two cells are shown on the left. Are they wild type or GCN4?

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

This paper provides new insight into the steps required to separate spindle poles in the earliest stages of building the spindle. The authors use the microtubule-anchoring pole component Spc110 to test the idea that the flexibility in pivoting microtubules is key. They use the tension sensor module that has been described in FRET exp (Grashoff) and biophysical approaches and theory to explain their results. This is one of the most interesting papers I have seen in this field for years. It is an elegant study and well executed.
I have only a very minor point. They write that radius of a mt is 25 nm (end of thermal fluctuation analysis). I thought 25 nm was the diameter. I presume this is a typo, but they should double check what they used in their code.

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE

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December 4, 2020

Dr. Melissa Gardner, PhD
Monitoring Editor
Journal of Cell Biology

Dear Melissa,

Thank you very much for serving as Monitoring Editor for our manuscript, now entitled, "Microtubule pivoting enables mitotic spindle assembly in *S. cerevisiae*." We are grateful to the reviewers for their careful consideration and helpful suggestions, which we believe have significantly improved our paper. Point-by-point replies to all of their comments and summaries of the major changes we made in response can be found below. We hope that with these changes the work will now be considered acceptable for publication in the *Journal of Cell Biology*.

Sincerely,

Charles (Chip) Asbury

Trisha N. Davis

Kimberly Fong

Reviewer #1 (their full comments are recopied in black; our responses are printed in blue):

Assembly of bipolar mitotic spindles accompanies spindle pole separation. A prerequisite for this process is formation of pole-to-pole antiparallel microtubules that nucleate from each pole. Although our understanding of the molecular pathways leading to spindle assembly has been substantially improved during the last decade or so, details of the initial step that promotes antiparallel microtubule formation and molecules involved in this process are still largely elusive. Previous work performed in fission yeast showed that microtubule pivoting would be crucial for bipolar spindle formation, but how this is achieved at the molecular level is not fully understood. In this manuscript, Fong et al. have addressed this unsolved question in budding yeast, an organism that is supposed to need a strict regulatory system for this process.

The authors picked up Spc110, a component of the spindle pole body (SPB), for analysis of microtubule pivoting. Spc110 belongs to a widely conserved protein family, contains a long internal coiled-coil stretch and is known to function as a structural linker between the SPB and the microtubule minus-end-capping and nucleator complex (the gamma-tubulin ring complex). The idea behind this is that the long, internal coiled-coil stalk that contains several hinges/breaks might provide Spc110 with structural flexibility that promotes microtubule pivoting. Given this assumption, they first created four types of mutant strains that contain different Spc110 variants. CCΔ lacks two-thirds of the coiled-coil stalk (expected to be less flexible), GCN4 contains 33 amino acid residues derived from well-characterized, leucine zipper at the junction within CCΔ (expected to be stiff), SKIP4 contains a distinct, flexible hinge at the junction (expected to be flexible), and finally TSMOD replaces the tension sensor module for a 124-residue portion of the coiled coil stalk (expected to be very flexible). Then, SPBs from individual strains were purified and in vitro assay of microtubule pivoting performed. Interestingly, and consistent with the proposition, individual SPBs displayed the varied degrees of microtubule pivoting with the order of TSMOD, SKIP4, wild type, GCN4 and CCΔ. Having obtained the in vitro data for individual Spc110 constructs, the authors next characterized in vivo behaviors of these strains, in particular focusing on the timing of SPB separation upon onset of mitosis. Largely in line with the data from in vitro assay, CCΔ exhibited substantial delay in SPB separation, while TSMOD showed almost the same timing, and GCN4 and SKIP4 displayed modest delay. More detailed analysis performed in wild type and GCN4 cells showed that the timing of SPB separation was indeed delayed in GCN4 by 7 min compared to wild type cells.

This study contains fairly interesting new findings on microtubule pivoting and provides new insight into the initial stage of spindle assembly. In particular, in vitro assay is beautifully performed and well presented. I would like to raise a couple of points, and I hope that the authors could address them.

We sincerely thank the reviewer for their thoughtful consideration of our work. We are delighted that they find our *in vitro* results interesting and well presented. We hope the changes we have made to the manuscript, detailed below, will address satisfactorily the additional points they have raised.

Major comments:

1) Basic characterization of Spc110 mutant strains
Compared to the results from in vitro assay, those of microtubule pivoting in cells are less convincing. I understand the visualization of microtubule pivoting would be technically challenging, but a few more data on Spc110 mutant proteins might be required. First, the authors seem to postulate that the amount of individual Spc110 mutant proteins is the same as that of wild type, but no data is presented. I think it is important to show this by simple immunoblotting or quantification of individual Spc110 proteins on SPBs. Second, it is important to show recruitment of the gamma-tubulin complex to the SPB is not compromised in each mutant.

We thank the reviewer for raising this important point. When we designed our mutant Spc110 constructs, we were careful to avoid any changes to the C- and N-terminal portions through which Spc110 binds to SPBs and

recruits the γ-tubulin complex, respectively. Since all four mutants support viability as the sole copy of Spc110, and three of the four support normal cell growth rates, we had assumed that the fundamental linker function of Spc110 was preserved. However, to address the reviewer's concern directly, we have now performed an additional test, the results of which confirm that normal levels of γ-tubulin complex are indeed recruited to SPBs in all four mutant strains. Briefly, we constructed strains carrying a fluorescent-tagged component of the γ-tubulin complex, Spc97-GFP, in combination with our mutant Spc110 constructs or with wild type Spc110 as a control. Fluorescence intensities of the SPBs in all the strains were indistinguishable, indicating consistent recruitment of γ-tubulin complex to the SPBs. And because the majority of γ-tubulin complex is recruited to SPBs via Spc110, the consistent levels of γ-tubulin complex also indicate that the levels of SPB-bound Spc110 are essentially normal as well. Data from these fluorescence measurements are now included as Supplemental figure 1D, and are described in the text, lines 126 to 128, on page 5.

2) Observation of spindle morphology
GCN4 cells display 7 min delay in the timing of SPB separation (Figure 4B). It would be nice to show live imaging of spindle morphology in parallel with wild type cells. Is there any difference in spindle intensity between GCN4 and wild type cells? If microtubule pivoting is defective in GCN4, spindle intensity during this delay period might be reduced.

If we understand correctly, the reviewer is asking for a comparison of tubulin distributions in wild type versus GCN4 mutant cells, based on live imaging specifically during the seven-minute period after release from G1/S (using the double synchronization protocol). A majority of wild type cells separated their SPBs during this seven-minute period, whereas only a minority of the mutant GCN4 did so (as shown in Figure 4B). We agree that different microtubule arrangements are expected, particularly when comparing cells with separated versus unseparated SPBs. However, the spatial resolution of light microscopy is insufficient to distinguish individual nuclear microtubules in *S. cerevisiae* due to the density of the filaments and their short lengths. Thus, before SPB separation, which is the situation when pivoting is expected to play a crucial role, the tubulin distribution appears as a single 'blob'. It is not clear to us that the reduced pivoting in GCN4 would reduce the intensity of this blob. It might affect the shape, but we doubt a shape change near the limit of optical resolution could provide much useful information about the arrangement of microtubules. Also, while our double block-release protocol achieved excellent synchrony in liquid culture, we have yet to achieve such synchrony in our attempts thus far to image cells growing live on agar pads after release from even a simple α-factor arrest. Because the proposed experiment is technically quite challenging and, in our view, would not necessarily provide important new insights, we believe it is beyond the scope of the present paper.

Minor comments:

3) the title
The current title may not be the best choice, as it looks like a review article. I suggest a new title such as "Microtubule pivoting promotes mitotic spindle assembly in budding yeast"

We thank the reviewer for this suggestion. We agree that the original title could have been misinterpreted as that of a review article rather than an original research paper. We have revised the title to, "Microtubule pivoting enables mitotic spindle assembly in *S. cerevisiae.*"

4) Figure 1C, the legend
Please explain black arrowhead.

We thank the reviewer for this comment. The arrowhead is now explained in the legend of Figure 1C (lines 751 – 752, page 26).

5) Figure 4, the legend
"cdc28-as1 cells (gold) versus wild type cells (blue)" should be "cdc28-as1 containing GCN4 cells (gold) versus cdc28-as1 containing wild type Spc110 cells (blue)".

Thank you for pointing out this mistake. The caption of Figure 4 has been corrected (lines 786, and 790 – 791, page 27).

6) Figure 4B
Image of two cells are shown on the left. Are they wild type or GCN4?

The images show cells with wild type Spc110, and this information is now stated in the legend of Figure 4B (line 795, page 27).

Reviewer #2 (their full comments are reprinted in black; our responses are printed in blue):

This paper provides new insight into the steps required to separate spindle poles in the earliest stages of building the spindle. The authors use the microtubule-anchoring pole component Spc110 to test the idea that the flexibility in pivoting microtubules is key. They use the tension sensor module that has been described in FRET exp (Grashoff) and biophysical approaches and theory to explain their results. This is one of the most interesting papers I have seen in this field for years. It is an elegant study and well executed.

We thank the reviewer for their interest in our work. We are delighted that they find it to be elegant and well executed.

I have only a very minor point. They write that radius of a mt is 25 nm (end of thermal fluctuation analysis). I thought 25 nm was the diameter. I presume this is a typo, but they should double check what they used in their code.

We thank the reviewer for catching this typo, which we have corrected in the revised manuscript (line 498, page 17). The correct number was used in our calculations, which are provided as an excel spreadsheet – equations included (Supplemental table 4).

December 10, 2020

RE: JCB Manuscript #202007193R

Dr. Charles L Asbury
University of Washington School of Medicine
Physiology & Biophysics
1959 NE Pacific St, HSB G-424
Box 357290
Seattle, Washington 98195-7290

Dear Dr. Asbury:

Thank you for submitting your revised manuscript entitled "Microtubule pivoting enables mitotic spindle assembly in S. cerevisiae". 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 Reports is <20,000, not including spaces. Count includes title page, abstract, introduction, combined results and discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Reports may have up to 5 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.

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 (either in the figure legend itself or 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."

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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 in the text for readers who may not have access to referenced manuscripts. *

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

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. Reports may have up to 3 supplemental display items (figures and tables). 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.

11) 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.

12) 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."

13) 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.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Melissa Gardner
Monitoring Editor

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Senior Scientific Editor

Journal of Cell Biology