Three interacting regions of the Ndc80 and Dam1 complexes support microtubule tip-coupling under load

Rachel Flores, Zachary Peterson, Alex Zelter, Michael Riffle, Charles Asbury, and Trisha Davis

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

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August 6, 2021

Re: JCB manuscript #202107016

Dr. Trisha Davis
University of Washington School of Medicine
Biochemistry
1705 NE Pacific Street
Seattle, WA 98195-7350

Dear Dr. Davis,

Thank you for submitting your manuscript entitled "Distinct Ndc80-Dam1 complex interactions support coupling to growing versus shortening microtubules". The manuscript has been evaluated by two expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication of the submitted manuscript in JCB.

You will see that the reviewers have numerous and substantial concerns. Reviewer #1 has specific concerns and questions related to the experimental analysis that all seem valid and important to address. Reviewer #2, while also raising specific points, questions the level of advance presented by the work for the JCB audience. After discussing the reviewer feedback, our editorial assessment is that a significantly revised study that addresses all of the specific reviewer comments and is restructured to emphasize the significance of the findings to a broad cell biology audience has the potential to be of interest to the readership of JCB. We appreciate that addressing the points raised by the reviewers will require substantial additional effort and likely extend beyond a typical revision period. If you wish to expedite publication of the current version, it may be best to pursue publication at another journal.

If, after considering the reviewer feedback you and your co-authors would like to resubmit a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

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 the reviewer comments further once you've had a chance to consider the points raised in this letter. 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,

Arshad Desai, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

In the manuscript by Flores et al. entitled "Distinct Ndc80-Dam1 complex interactions support coupling to growing versus shortening microtubules", the authors reconstitute kinetochore-microtubule interface of the budding yeast using purified components - microtubules, Dam1 complex and Ndc80 complex. To grasp the complexity of the interaction interfaces between the three components, the authors test an impressive number of experimental conditions. Building on their previous paper (Kim et al, eLife 2017) reporting three regions in Ndc80 and three regions in Dam1 as possible interaction points between the two complexes residing on microtubules, the authors now probe these interactions under force. Using sophisticated optical tweezers instrumentation, the authors perform two signature Asbury lab experiments: the rupture force assay, in which they probe the force at which the bead-microtubule connection breaks, and the force-clamp assay, in which the dynamic microtubule end is the

target of a constant force acting through the bead-bound Ndc80 and Dam1 complexes. Based on these assays, and on their previously published work, the authors conclude that two of the three Dam1-interacting regions in Ndc80 (regions A and B) support interactions of the beads with both growing and shortening microtubule ends, while the third Ndc80 region (C) only plays a role during microtubule shortening.

The manuscript constitutes a significant advance in our understanding of the (phospho-)regulation of kinetochore-microtubule interface. Results are presented in a very detailed way. I applaud the rigorously reported statistical analyses which include t-test results for (almost) all possible combinations of biochemical interactions tested, as well as means and confidence intervals for biological replicates in the rupture force assays. Nevertheless, I have several critical concerns that, in my opinion, prevent the publication of this paper.

My main concern is related to the stoichiometry of the reported interactions. In a previous paper by the Davis and Asbury labs (Kim et al, eLife 2017), a single Ndc80 complex was reported to bridge two full Dam1 rings. Looking at Figure 3A and Figure 6 in the current manuscript, I came under an impression that the authors interpret their optical tweezers data in the same way: a single WT Ndc80 complex binds two full WT Dam1 rings while the whole construct follows the dynamic microtubule end. However, this important issue is not addressed clearly in the manuscript. Moreover, the data presented here can be interpreted in other ways, which the authors do not discuss; namely (1) that a single Ndc80 complex binds one full Dam1 ring that shifts its position between binding regions A, B and C in Ndc80 depending on force or polymerization status of the microtubule end; and (2) that Ndc80 regions A, B and C recruit incomplete Dam1 rings or Dam1 rings of varying completeness. Specifically, these points require more attention:

1. The authors should address Ndc80/Dam1 stoichiometry more clearly when describing the data in Figure 1B. How many Ndc80 complexes and Dam1 subunits are present in the interaction being put under force? What is the contribution of a full Dam1 ring to the rupture force? For example, should the difference between rupture force in conditions "WT Ndc80 + WT Dam1" and "Ndc80 mutant A + WT Dam1" be interpreted as the loss of one of the two Dam1 rings, as suggested by the diagram on the left in Figure 1B, or both rings, if there were two Dam1 rings per Ndc80 in the first case? Or is an alternative interpretation true: there was one (complete or incomplete) Dam1 ring bound per Ndc80, and it is lost upon mutation in Ndc80 region A, and maybe partially lost upon mutations in regions B and C?
2. How do the authors interpret the lack of difference in rupture force when comparing Dam1 phosphorylated at regions ABC vs AB and ABC vs AC, especially in light of a statistically significant difference observed for phosphorylation in Dam1 regions ABC vs BC? Does it mean that the only force-resistant interaction region in Dam1 is dephosphorylated region A(Dam1)?
3. How do the authors interpret the difference in rupture force between WT Ndc80 alone (Figure 1B, top row, median rupture force 3.2 pN) and WT Ndc80 + fully phosphorylated Dam1 (Figure 2B, top row, median rupture force 5.5 pN)? Is the increase in the rupture force between these two datasets indicative of a Dam1-Ndc80 interaction interface that is not disrupted upon Ipl1 treatment?

My second concern is regarding the pre-loading at 1 pN in rupture force assays, and the way the authors chose to present it in the manuscript. The data presented in main figures are filtered: data on beads that did not hold up at 1 pN are only found in the supplement. However, these missing data are critical for the correct interpretation of the results. For example, as the authors mention themselves, Ndc80 with a mutation in region A produces weaker attachments than WT Ndc80: detachment at 1 pN is twice more frequent for Ndc80 mutant A (Figure S1A, $p < 0.001$ according to Table S1). Therefore, the apparent lack of difference between rupture force with WT Ndc80 and Ndc80 mutant A (Figure 1B, $p = 0.058$ according to Table S2) is misleading: only beads that survived the pre-loading are compared, which leads to enrichment of strong attachments in the analyzed Ndc80 mutant A sample. I suggest including the beads that did not survive the pre-loading in the main figures, just like the beads that escaped the trap, and discussing more clearly the limitations of the rupture force assay.

My third concern: the authors should discuss previously published evidence that conflicts with their interpretations. In two papers from the Bloom and Salmon labs (Joglekar et al., Nat Cell Biol 2006 and Joglekar et al., 2008 J Cell Biol), the copy numbers of Dam1 and Ndc80 complexes measured at budding yeast kinetochores were reported to be in a ratio of 2 and 1.5-2 Dam1 per Ndc80, respectively, with Dam1 present in amounts sufficient to form only one ring (16 and 16-20 copies, respectively). Not only these reports estimate the Dam1/Ndc80 ratio at an order of magnitude below what is proposed here, but they also suggest that the absolute quantity of Dam1 at the kinetochore is inconsistent with a double ring per microtubule attachment site. Similarly, in a paper from the McIntosh lab (Grishchuk et al., PNAS 2008 https://doi.org/10.1073/pnas.0801811105), Dam1 oligomers bigger than one ring were reported to stop microtubule shortening, conflicting with the authors' interpretations. None of these papers are cited or discussed.

Finally, several technical notes:

- What are the detachment rates for Ndc80 alone, without addition of Dam1 complex as presented in Figures 4 and 5?
- Please provide statistical tests for data presented in Figures 4 and 5.
- Please provide statistical tests for data presented in Figures S1BC and S5B. How different are the values between and within the biological replicates?

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

In the manuscript, "Distinct Ndc80-Dam1 complex interactions support coupling to growing versus shortening microtubules," Flores et al. investigate the molecular interactions between the budding yeast Ndc80 and Dam1 protein complexes, which are both important for ensuring accurate chromosome segregation during mitosis. Previous work from the Asbury and Davis labs found that three subunits of the Dam1 complex (Dam1p, Ask1p and Spc34p) interact with three different regions on the Ndc80 protein. Disrupting these interactions by inserting amino acids into the relevant regions of Ndc80p reduces interaction between the two complexes in vitro and impairs chromosome segregation in cells. Furthermore, they demonstrated that phosphorylation of the three Dam1 components by Ipl1 kinase inhibits the interaction between the Dam1 and Ndc80 complexes (Kim et al., 2017). In the current study, the authors build on their long-standing expertise in this area and ask if these interactions are important for supporting load-bearing attachments to dynamic microtubules and if all three interactions participate equally in coupling to growing vs. shortening microtubules. To test this, the authors purified wild-type and mutant budding yeast Ndc80 and Dam1 complexes for use in laser trapping experiments. They demonstrate that two of the interactions are important for maintaining load-bearing attachments to growing microtubule ends, and all three interactions are important for load-bearing coupling to shortening microtubule ends. This is a well-constructed study and the data convincingly support the authors main conclusion. While this is the case, there is not much new insight into how these three known Ndc80-Dam1 points of interaction are coordinated during dynamic microtubule behavior, how Ipl1 phosphorylation may regulate a subset of interactions during growth vs. shortening phases, or how phosphorylation differentially regulates Ndc80-Dam1 interaction affinity vs. load-bearing ability. As such, I feel that the study may be of interest primarily to a somewhat narrow slice of the yeast mitosis field. Additional comments are provided below.

(1) The authors make the point that phosphorylating any of the three Dam1 interaction sites inhibits Ndc80 binding. Thus, it is not clear why there is such a modest effect on load-bearing tip-coupling to growing MT ends in Figure 2 vs. Figure 1, where the interactions are impaired using amino acid insertions in Ndc80p.

(2) Figure 3, Panel A seems is a illustration showing that MTs switch from growth to shortening and Ndc80 can become detached during either phase. I'm not sure a schematic is needed here (or I might be missing something). Perhaps a representation of the experimental method would be more helpful. In Panel B, the authors are showing examples of MT life history traces under either 1.5 pN or 3.5 pN of force, but in the text it is not clear what the reader is supposed to take home from this - are there certain trends that the authors want to point out with the different levels of force?

(3) In the paragraph starting, "In the absence of the Dam1 complex, very few Ndc80 complex coated beads could...," there is no figure call-out for the description of how the addition of Dam1 affects the ability of Ndc80-coated beads to track dynamic tips. (This may relate to the above point number 3?)

(4) In Figure 5, the authors show that insertions in Ndc80p affect the ability to remain attached to depolymerizing microtubules. Given that the more physiological mechanism for regulating Ndc80-Dam1 interactions is through phosphorylation (not amino acid insertion), it would be critical to do the experiment with the phosphorylated complexes.

UNIVERSITY of WASHINGTON
DEPARTMENT OF BIOCHEMISTRY
School of Medicine

December 1, 2021

Arshad Desai
Andrea L. Marat
Journal of Cell Biology
950 Third Ave., 2nd Floor
New York, NY 10022, USA

RE: Manuscript number: 202107016

Dear Arshad and Andrea:

Thank you for serving as Reviewing Editor and Senior Editor for our work. We are grateful for your efforts and those of the reviewers. In response to the many helpful comments, we have added a considerable amount of new data and made extensive revisions to the manuscript. Our point-by-point responses, detailing all the changes, are given on the following pages. We hope that with these changes the paper will be considered ready for publication in JCB

Sincerely,

Trisha N. Davis
Professor and Chair
Earl W. Davie/Zymogenetics Endowed Chair
Department of Biochemistry

Charles L. Asbury
Professor
Department of Physiology and Biophysics

In response to the reviewer's comments, we have performed extensive new experiments and completely reworked our paper. Two primary concerns were that we had not delved sufficiently into phosphoregulation and that we had made unjustified assumptions about the structural organization of the Dam1 complexes in our assays. Both concerns are thoroughly addressed in the revised version. Phosphoregulation is now a major focus, supported by a large set of new constant force measurements using Dam1 complexes phosphorylated at each of the three interacting regions. These new measurements were specifically requested by Reviewer 2 and challenging to perform. They revealed an unexpected behavior that we believe will enhance the novelty and impact of our work as detailed below and in the paper. The data show clearly that Dam1 complexes interact with Ndc80 complexes via all three regions, since all three contribute to the formation of a persistent, load-bearing tip-coupler. However, the exact structural organization of the Dam1 complexes in our laser trap assays is unknown and we apologize that this uncertainty was not made clearer in our original manuscript – it is now stated explicitly in the revised version. We also updated our schematics to depict multiple different configurations of Dam1 complexes. Point-by-point responses to all the reviewer's comments are given below. We are grateful for their advice and consideration of our work.

Reviewer 1:

In the manuscript by Flores et al. entitled "Distinct Ndc80-Dam1 complex interactions support coupling to growing versus shortening microtubules", the authors reconstitute kinetochore-microtubule interface of the budding yeast using purified components - microtubules, Dam1 complex and Ndc80 complex. To grasp the complexity of the interaction interfaces between the three components, the authors test an impressive number of experimental conditions. Building on their previous paper (Kim et al, eLife 2017) reporting three regions in Ndc80 and three regions in Dam1 as possible interaction points between the two complexes residing on microtubules, the authors now probe these interactions under force. Using sophisticated optical tweezers instrumentation, the authors perform two signature Asbury lab experiments: the rupture force assay, in which they probe the force at which the bead-microtubule connection breaks, and the force-clamp assay, in which the dynamic microtubule end is the target of a constant force acting through the bead-bound Ndc80 and Dam1 complexes. Based on these assays, and on their previously published work, the authors conclude that two of the three Dam1-interacting regions in Ndc80 (regions A and B) support interactions of the beads with both growing and shortening microtubule ends, while the third Ndc80 region (C) only plays a role during microtubule shortening.

The manuscript constitutes a significant advance in our understanding of the (phospho-)regulation of kinetochore-microtubule interface. Results are presented in a very detailed way. I applaud the rigorously reported statistical analyses which include t-test results for (almost) all possible combinations of biochemical interactions tested, as well as means and confidence intervals for biological replicates in the rupture force assays. Nevertheless, I have several critical concerns that, in my opinion, prevent the publication of this paper.

We thank the reviewer for their positive comments and constructive criticisms. Our new measurements reveal that phosphorylation of region C on the Dam1 complex reduces tip-coupling stability specifically during long-term application of constant force, even though it does not affect short-term rupture strengths measured on growing tips. We believe this unique influence on long-term stability has interesting implications for error correction, as mentioned below and in our revised discussion. We hope we have adequately addressed each of your concerns and that you will find the revised manuscript suitable for publication.

ESSENTIAL REVISIONS:

1. The authors should address Ndc80/Dam1 stoichiometry more clearly when describing the data in figure 1B. How many Ndc80 complexes and Dam1 subunits are present in the interaction being put under force? What is the contribution of a full Dam1 ring to the rupture force? For example, should the difference between rupture force in conditions “WT Ndc80 + WT Dam1” and “Ndc80 mutant A + WT Dam1” be interpreted as the loss of one of the two Dam1 rings, as suggested by the diagram on the left in Figure 1B, or both rings, if there were two Dam1 rings per Ndc80 in the first case? Or is an alternative interpretation true: there was one (complete or incomplete) Dam1 ring bound per Ndc80, and it is lost upon mutation in Ndc80 region A, and maybe partially lost upon mutations in regions B and C?

The exact structural organization of Dam1 complexes in our laser trap assays is unknown, and we apologize that our original manuscript was not clearer on this point. Our data do show clearly that Dam1 complexes interact with all three regions of the Ndc80 complex, since all three contribute to the formation of a persistent, load-bearing tip-coupler. We have added a new schematic that is agnostic regarding the arrangement of the Dam1 complex but instead shows a map of the regions of the Dam1 complex that interact with the Ndc80 complex based on recent structures of the two complexes from the Harrison lab (Figure 1A).

Regarding the stoichiometry of the interaction, our original schematics depicting two complete Dam1 rings bound to a single Ndc80 complex were unintentionally confusing and we have updated our schematics to show multiple Ndc80 complexes with a variety of different arrangements of Dam1 complexes (in Figures 1B, 3A, and 3B). The schematic originally included as Figure 5 has been removed.

Based on the density at which we decorated our beads, and on simple geometric considerations, we expect many Ndc80 complexes to interact simultaneously with the microtubule tip, up to a maximum of about ninety. The upper-bound estimate for the number Ndc80 complexes that could simultaneously interact with the microtubule tip is explained (on pg. 5 of results, and pg. 13 in methods). We cannot determine the precise number or arrangement of Dam1 complexes because our laser trap assays only allow visualization of the beads (coated with Ndc80 complexes) and the microtubules, not the Dam1 complexes. This uncertainty about the arrangement of Dam1 complexes is now stated explicitly in the revised results, on pg. 5: “*Free Dam1 complexes...were not visible in the trapping microscope so their precise structural arrangement at the Ndc80-microtubule interface was unknown.*”

2. How do the authors interpret the lack of difference in rupture force when comparing Dam1 phosphorylated at regions ABC vs AB and ABC vs AC, especially in light of a statistically significant difference observed for phosphorylation in Dam1 regions ABC vs BC? Does it mean that the only force-resistant interaction region in Dam1 is dephosphorylated region A(Dam1p)?

We thank the reviewer for this question. Phosphorylating B^{Ask1p} alone causes a defect in rupture strength (Figure 3, Table S3), indicating that region A^{Dam1p} cannot be the only force-resistant interaction region of the Dam1 complex. However, we agree it is interesting that phosphorylating both A^{Dam1p} and B^{Ask1p} together causes a strength defect no worse than phosphorylating A^{Dam1p} alone. This observation suggests that A and B act redundantly, as we now mention in the revised results, on pg. 6: “*Phosphorylating regions A^{Dam1p} and B^{Ask1p} together caused no further weakening compared to phosphorylation of A^{Dam1p} alone, suggesting redundancy in the load-bearing function of regions A^{Dam1p} and B^{Ask1p}* ”. Because phosphorylating region C^{Spc34p} alone caused no defect in

rupture strength, despite stoichiometric levels of phosphorylation, it is not surprising that phosphorylating both A^{Dam1p} and C^{Spc34p} together was no worse than phosphorylating A^{Dam1p} alone.

3. How do the authors interpret the difference in rupture force between WT Ndc80 alone (Figure 1B, top row, median rupture force 3.2pN) and WT Ndc80 + fully phosphorylated Dam1 (Figure 2B, top row, median rupture force 5.5pN)? Is the increase in the rupture force between these two datasets indicative of a Dam1-Ndc80 interactions interface that is not disrupted upon Ipl1 treatment?

We believe the most likely explanation for the partial strength defect is that regions A^{Dam1p} and B^{Ask1p} were not fully phosphorylated, as we reported in Table 1 and Supplemental Figure S3. This interpretation is explicitly suggested in the revised results on pg. 6: "*When measured in the presence of Dam1 complex phosphorylated by Ipl1p at all three regions, A^{Dam1p}, B^{Ask1p}, and C^{Spc34p}, the median strength of Ndc80 complex-based couplers was 5.5 pN (Fig. 2 B). This value is higher than the strength of couplers based on wild-type Ndc80 complex alone (3.2 pN, Fig. 1 C), which indicates that our phosphorylation of all three regions of the Dam1 complex did not completely disrupt its load-bearing interactions with the Ndc80 complex, perhaps because we were unable to fully saturate all the phosphorylation sites on Dam1p and Ask1p (Table 1, Fig. S3).*"

4. My second concern is regarding the pre-loading at 1 pN in rupture force assays, and the way the authors chose to present in the manuscript. The data presented in main figures are filtered: data on beads that did not hold up at 1 pN are only found in the supplement. However, these missing data are critical for the correct interpretation of the results. For example, as the authors mention themselves, Ndc80 with a mutation in region A produces weaker attachments that WT Ndc80: detachments at 1 pN is twice more frequent for Ndc80 mutant A (Figure S1A, $p < 0.001$ according to Table S1). Therefore, the apparent lack of difference between rupture force with WT Ndc80 and Ndc80 mutant A (Figure 1B, $p=0.058$ according to Table S2) is misleading: only beads that survived the pre-loading are compared, which leads to enrichment of strong attachments in analyzed Ndc80 mutant A sample. I suggest including the beads that did not survive the pre-loading in the main figure, just like the beads that escaped the trap, and discussing more clearly the limitations of the rupture force assay.

We agree that our original presentation was confusing, and we thank the reviewer for this suggestion. In the revised manuscript, we now include plots showing the fractions of beads that did not survive the 1 pN preload, and also the fractions of beads that reached the load-limit of the trap without rupturing, in the main Figures (1C and 2B), juxtaposed beside the rupture force plots. We agree this is a better, more comprehensive presentation of the data. Incorporating the left-censored data (i.e., the beads that failed to hold the 1 pN preload) does not change our primary conclusions that interaction regions A and B contribute to rupture strength, while region C is dispensable in the rupture strength assay.

5. My third concern: the authors should discuss previously published evidence that conflicts with their interpretations. In two papers from the Bloom and Salmon labs (Joglekar et al., Nat Cell Biol 2006 and Joglekar et al., 2008 J Cell Biol), the copy numbers of Dam1 and Ndc80 complexes measured at budding yeast kinetochores were reported to be in a ratio of 2 and 1.5-2 Dam1 per Ndc80, respectively, with Dam1 present in amounts sufficient to form only one ring (16 and 16-20 copies, respectively). Not only these reports estimate the Dam1/Ndc80 ratio at an order of magnitude below what is proposed here, but they also suggest that the absolute quantity of Dam1 at the kinetochore is inconsistent with a double ring per microtubule attachment site. Similarly, in a paper from the McIntosh lab (Grishchuk et al., PNAS 2008), Dam1 oligomers bigger than one ring

were reported to stop microtubule shortening, conflicting with authors' interpretations. None of these papers are cited or discussed.

We certainly did not mean to suggest a Dam1/Ndc80 ratio 10-fold higher than the estimates from Joglekar et al. 2006, nor that we knew the structural arrangement of the Dam1 complexes in our assays. Because our schematic depicting two complete Dam1 rings associated with a single Ndc80 complex (formerly Figure 5) might have inadvertently given that impression, it has now been removed. We apologize for any confusion it caused. And as mentioned above in our response to point 1, we now state explicitly in the results (on pg. 5) that the arrangement of Dam1 complexes in our assays is unknown.

We agree that a brief review of the estimated numbers of Dam1 complexes per kinetochore *in vivo* will be valuable. This is now included in our revised introduction, on pg. 3, together with the appropriate citations: "*High-resolution molecular structures for key portions of the fungal Ndc80 and Dam1 complexes are now available (Valverde et al., 2016; Jenni and Harrison, 2018), but their precise arrangement at kinetochores in cells remains unclear. A recent study using cryo-electron tomography found many partial and full Dam1 complex rings near the tips of spindle microtubules and, occasionally, two rings per microtubule (Ng et al. 2018). Efforts to quantify the average number of Dam1 complexes per kinetochore have reported values between 16 and 32 (Joglekar et al. 2006; Lawrimore, Bloom, and Salmon 2011). The latter would be sufficient to form two complete rings.*"

Because we are not claiming to have complete rings in our assays, the results of Grishchuk *et al*. 2008 do not conflict with our interpretations. We also note that Grishchuk *et al*. only observed complete stalling (stopping) of microtubule disassembly by multiple Dam1 rings in the absence of free tubulin. In our experience, free tubulin weakens the binding of Dam1 complexes to microtubules (Graczyk and Davis, 2011). The free tubulin present in our trap experiments (~20 μM) might therefore have reduced the amount of Dam1 complex on our microtubules relative to the experiments of Grishchuk *et al*. or, in principle, it could have allowed multiple rings to track with the disassembling tips in our experiments without causing the tips to completely stall.

6. Finally, several technical notes:
a. What are the detachment rates for Ndc80 alone, without addition of Dam1 complex as presented in Figures 4 and 5?

We thank the reviewer for their interest in the lifetime experiments with couplers based on Ndc80 complex alone, in the absence of Dam1 complex. Unfortunately, because the concentration of Ndc80 complex on the beads was relatively low in these experiments, the fraction of beads that gave measurable tip-coupling when only Ndc80 complex was present was so low that we could not calculate detachment rates for them. This very low tracking frequency is reported explicitly in the revised results, on pg. 7: "*In the absence of the Dam1 complex, very few Ndc80 complex-coated beads could track persistently with shortening microtubule tips under the applied force of 1.5 pN (only 3 out of 41 beads tested, or 7%) and none could track at 3.5 pN (0 out of 33 beads tested).*"

b. Please provide statistical tests for data presented in Figure 4 and 5.

The data originally shown in Figures 4 and 5 have been reorganized in the revised manuscript. All the detachment rate data are now combined in Figure 4 and all the switch rate data (i.e., catastrophe and rescue rates) are moved into Supplement Figure S6. Supplemental Table S6 includes the statistical comparisons for Figures 4 and S6, as requested.

c. Please provide statistical tests for data presented in Figure S1BC and S5B. How different are the values between and within the biological replicates?

Supplemental Tables S2 and S4 now provide the statistical tests comparing biological replicates, as requested.

Reviewer 2:

In the manuscript, "Distinct Ndc80-Dam1 complex interactions support coupling to growing versus shortening microtubules," Flores et al. investigate the molecular interactions between the budding yeast Ndc80 and Dam1 protein complexes, which are both important for ensuring accurate chromosome segregation during mitosis. Previous work from the Asbury and Davis labs found that three subunits of the Dam1 complex (Dam1p, Ask1p and Spc34p) interact with three different regions on the Ndc80 protein. Disrupting these interactions by inserting amino acids into the relevant regions of Ndc80p reduces interaction between the two complexes in vitro and impairs chromosome segregation in cells. Furthermore, they demonstrated that phosphorylation of the three Dam1 components by Ipl1 kinase inhibits the interaction between the Dam1 and Ndc80 complexes (Kim et al., 2017). In the current study, the authors build on their long-standing expertise in this area and ask if these interactions are important for supporting load-bearing attachments to dynamic microtubules and if all three interactions participate equally in coupling to growing vs. shortening microtubules. To test this, the authors purified wild-type and mutant budding yeast Ndc80 and Dam1 complexes for use in laser trapping experiments. They demonstrate that two of the interactions are important for maintaining load-bearing attachments to growing microtubule ends, and all three interactions are important for load-bearing coupling to shortening microtubule ends. This is a well-constructed study and the data convincingly support the authors main conclusion. While this is the case, there is not much new insight into how these three known Ndc80-Dam1 points of interaction are coordinated during dynamic microtubule behavior, how Ipl1 phosphorylation may regulate a subset of interactions during growth vs. shortening phases, or how phosphorylation differentially regulates Ndc80-Dam1 interaction affinity vs. load-bearing ability. As such, I feel that the study may be of interest primarily to a somewhat narrow slice of the yeast mitosis field. Additional comments are provided below.

We appreciate the reviewer's comments and positive feedback. In response, we have performed a large set of additional, challenging experiments measuring the behavior of the phosphorylated complexes under constant force. These new data reveal an unexpected behavior with implications for error correction *in vivo*. The manuscript has been extensively revised to incorporate the new results and to discuss their relevance for error correction. With these changes, we believe the work will appeal broadly to cell biologists and biophysicists interested in mitotic error correction across eukaryotes.

1. The authors make the point that phosphorylating any of the three Dam1 interactions sites inhibits Ndc80 binding. Thus, it is not clear why there is such modest effect on load-bearing tip-coupling to growing MT ends in Figure 2 vs Figure 1, where the interactions are impaired using amino acid insertions in Ndc80p.

   As noted above, in our response to Reviewer 1, point 3, we believe the most likely explanation for the partial strength defect is that regions A^{Dam1p} and B^{Ask1p} were not fully phosphorylated, as reported in Table 1 and Supplemental Figure S3. This interpretation is now explicitly suggested in the revised results (on pg. 6).

2. Figure 3, Panel A seems is an illustration showing that MTs switch from growth to shortening and Ndc80 can become detached during either phase. I'm not sure a schematic is needed here (or I might be missing something). Perhaps a representation of the experimental method would be more helpful. In Panel B, the authors are showing examples of MT life history traces under either 1.5 pN or 3.5 pN of force, but in the text it is not clear what the reader is supposed to take home from this – are these certain trends that the authors want to point out with the different levels of force?

   We thank the reviewer for their suggestions about Figure 3. We agree that depicting the force clamp assay would be helpful, especially since we used laser scissors to trigger microtubule shortening and facilitate the collection of many records of disassembly-driven movement. This procedure differs from most of our previous work, and the revised paper now includes a new illustration of it (Figure 3, panel A). In our view, depicting a microtubule tip switching between growth and shortening, and showing the two detachment pathways from each of these phases, is useful, particularly for readers less familiar with microtubule dynamics. We have therefore chosen to retain it (now Figure 3, panel B). However, we agree that the take-home message from our original presentation of life history traces was unclear. In the revised Figure 3, panel C, we now include representative traces recorded with all the measured combinations of Ndc80 and Dam1 complexes. Including all the combinations should help illustrate that detachments were more frequent when interaction regions A, B, or C were disrupted.

3. In the paragraph starting, "In the absence of Dam1 complex, very few Ndc80 complex coated beads could…," there is no figure call-out for the description of how the addition of Dam1 affects the ability of Ndc80-coated beads to track dynamic tips. (This may relate to the above point number 3?)

   We thank the reviewer for pointing out this omission, which was also noted by reviewer #1 in their point number 6 a, above. We now provide this information in the revised results, on pg. 7: "*In the absence of the Dam1 complex, very few Ndc80 complex-coated beads could track persistently with shortening microtubule tips under the applied force of 1.5 pN (only 3 out of 41 beads tested, or 7%) and none could track at 3.5 pN (0 out of 33 beads tested).*"

4. In Figure 5, the authors show that insertions in Ndc80p affect the ability to remain attached to depolymerizing microtubules. Given that the more physiological mechanism for regulating Ndc80-Dam1 interactions is through phosphorylation (not amino acid insertion), it would be critical to do the experiment with the phosphorylated complexes.

   We thank the reviewer for this suggestion and agree that further studies of the phosphorylated Dam1 complexes would be interesting and important. We therefore performed a large set of new measurements examining how the phosphorylated complexes behave under constant force conditions. These were challenging experiments, requiring many hours of additional data collection (as detailed in Supplementary Table S5). They revealed an unexpected behavior that we believe will enhance the novelty and impact of our work: Phosphorylating any of the three interacting regions reduces the long-term stability of tip-coupling (as shown in the yellow shaded region of Figure 4), even though phosphorylation of region C had no affect on short-term rupture strength (Figure 2B). This behavior with the phospho-C Dam1 complex falls strikingly outside the behaviors we measured with all the other combinations of complexes (as now shown in Figure 5). We think this differential regulation of long-term stability versus short-term strength has fascinating implications for mitotic error correction, as we now discuss on pg. 10: "*This specific reduction in long-term stability without loss of short-term strength could have interesting and important consequences for error correction* in vivo*. An initially unattached kinetochore is relaxed and therefore expected to be readily phosphorylated by Ipl1p/Aurora B. Nevertheless, when it*

establishes a new, correctly bioriented attachment, it must withstand the forces of biorientation long enough for the tension to halt Aurora B phosphorylation while dephosphorylation occurs and stabilizes the attachment (Khodjakov and Pines, 2010). The ability of attachments in which C^{Spc34p} is phosphorylated to survive high force might help to preserve bioriented attachments long enough for their dephosphorylation to occur."

February 17, 2022

RE: JCB Manuscript #202107016R-A

Dr. Trisha Davis
University of Washington School of Medicine
Biochemistry
1705 NE Pacific Street
Seattle, WA 98195-7350

Dear Dr. Davis:

Thank you for submitting your revised manuscript entitled "Interactions between three distinct regions of the Ndc80 and Dam1 complexes support coupling to dynamic microtubule tips under load", as well as for your response to the final remaining reviewer concerns as outlined below. We have discussed your response and re-revised manuscript with reviewer #1. While this reviewer continues to disagree with you on your interpretation of some of these points, the reviewer is supportive of publication. Therefore, 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:

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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 abstract, introduction, results, discussion, and acknowledgments. Count does not include title page, figure legends, materials and methods, 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. *

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."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

* Your current title is 132 characters, please provide a shortened alternative *

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.

* We encourage adding text and citations referencing the complementary work of additional groups to place this work in context of analysis of Aur B regulation of kinetochore-microtubule attachments across multiple systems. *

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. 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."

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

Sincerely,

Arshad Desai, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

In a revised manuscript by Flores et al entitled "Interactions between three distinct regions of the Ndc80 and Dam1 complexes support coupling to dynamic microtubule tips under load", the authors investigate the biophysics of a budding yeast kinetochore-microtubule attachment. Having previously established three regions in kinetochore complexes Ndc80 and Dam1 as potential interaction interfaces between these complexes, the authors now perturb these interfaces and perform optical trapping assays to probe the attachment strength between a bead coated with Ndc80 and Dam1 complexes, and dynamic microtubules. The revised version of the manuscript contains new data on the effects of Ipl1 phosphorylation on Dam1:Ndc80 coupling to the microtubule dynamics under constant force.

I congratulate the authors on an improved version of Figure 1 and accompanying description of results, which make it much clearer that Ndc80 with mutations in region A is somewhat dysfunctional. Unfortunately, this makes the experiments with Ndc80 mutant A + Dam1 complex less interesting, because it becomes difficult to disentangle the effects of Ndc80 mutant A folding, Ndc80 mutant A microtubule binding and Ndc80 mutant A : Dam1 interaction. I also note a more adequate Discussion. However, some of my comments to the initial manuscript version still remain in place after I've read the new version. I struggled particularly when reading a dense and somewhat confusing description of results on p. 6. Below are my comments to the new manuscript, which taken together prevent me from recommending this manuscript for publication.

1. My comment to the initial MS regarding the stoichiometry of the reported interactions is still standing. Phosphorylation of Dam1 by Ipl1 was previously reported to affect the oligomerization of Dam1, initially in Wang et al NSMB 2007, later by the authors themselves in Zelter et al Nat Comm 2015. This possibility is not discussed in the manuscript at all. Impaired Dam1 ring formation could affect the stability of bead-microtubule connection in ways similar to the ones which the authors attribute to the disruption of Dam1-Ndc80 connection (see for example, their paper by Umbreit et al. Nat Comm 2014, where presence of oligomerization-deficient Dam1 complex led to a decrease in rupture force of Ndc80/Dam1 coated beads). Coupled with incomplete Dam1 phosphorylation and unknown ratios of Ndc80 and Dam1 which apparently also vary between force ramp and force clamp assays, this complicates the correct interpretation of the authors' results.

2. In response to my comment to the initial MS regarding an almost 2-fold difference in rupture force comparing Ndc80 alone and Ndc80 + phosphorylated 6A-Dam1, the authors now report two additional sites in Dam1p (S31 and S311) as possible interaction points which were previously disregarded. However, they go on to state on p. 6 that "Phosphorylation at these

residues did not affect the strength of tip-couplers". Therefore, my concern remains: the difference in rupture force between Ndc80 alone and Ndc80 + phosphorylated 6A-Dam1 indicates that there might be Ndc80-Dam1 interactions which the authors fail to disrupt by their phosphorylation treatment.

3. The authors suggest a redundancy in Dam1 regions A and B since phosphorylation in A+B is no weaker than A alone or B alone. If this were true, comparison of AB to A (n.s., Table S3) should give the same result as the comparison of AB to B ($p = 0.036$, Table S3). In other words, addition of A to B weakens the attachment, while addition of B to A does not. Therefore, my initial suggestion still stands: if the authors compare (de)phosphorylation of individual regions, like they do when describing their force clamp data, they should conclude that the only force-resistant region in Dam1 is region A. This is the only conclusion that is consistent with further comparisons: ABC to AB (n.s.), ABC to AC (n.s.), but significant difference between ABC and BC.

4. As I mentioned in point 3 above, the authors are inconsistent when describing the effects of Dam1 phosphorylation. In sections reporting the rupture force assays they compare phosphorylated Dam1 with alanine substitutions to phosphorylation WT Dam1. In further sections reporting the experiments at constant force phosphorylated Dam1 with alanine substitutions is compared to phosphorylated 6A-Dam1. Making these two parts consistent with each other might help the clarity of presentation.

5. I do not share the authors' enthusiasm about the biological significance of the interaction region C for long-lived attachments. Rather, I think that this finding is not surprising: if the reduction in Ndc80-Dam1 affinity caused by disruption of the interaction region C is rather mild, it is quite trivial that one needs to wait longer to see the connection break. Given this explanation of the apparent discrepancy between the force-ramp and force-clamp data on region C, I do not see a reason to invoke any "interesting and important consequences for error-correction in vivo", as the authors do in the Discussion.

Minor point:
On p. 9 the authors write: "We demonstrate here that Ipl1p phosphorylation goes beyond regulating the affinity of the Dam1 and Ndc80 complexes for each other but also modulates the strength and stability of their interaction during tip coupling". I did not find the evidence supporting this statement in the manuscript, since the authors can not distinguish a breakage in Dam1:Ndc80 connection from breakage of Dam1-microtubule or Ndc80-microtubule connections.

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

The authors now include additional data that address how phosphorylation of three regions in Ndc80p affect the ability of NDC80C/Dam1C to maintain attachment to dynamic microtubules under load, which addresses an original major concern from this reviewer.

I still have one remaining point that I think is worth the author's attention:

In Figure 4, the authors include data from phosphorylated complexes under constant load. The authors conclude that during both microtubule growth and shortening, all three regions contribute to maintaining attachments to microtubules under load. I would agree that the data suggest all three regions modestly contribute to maintaining attachments during microtubule growth. During shortening, it is striking that region "A" seems to be the critical domain for this function (with a possible minor contribution from C or B). One question that I still have is how the authors can rule out that the effect is at least in part due to phosphorylation of the NDC80p complex affecting its ability to remain attached to shortening microtubules directly (and not solely a consequence of loss of Dam1C assistance?). In Figure 1, this mutant seems to be 3X less likely to bind microtubules under "pre-load" than wild-type NDC80p alone. The explanation to my question may be somewhere in the text, but perhaps the authors might reiterate this point?

Reviewer 1

In a revised manuscript by Flores et al entitled "Interactions between three distinct regions of the Ndc80 and Dam1 complexes support coupling to dynamic microtubule tips under load", the authors investigate the biophysics of a budding yeast kinetochore-microtubule attachment. Having previously established three regions in kinetochore complexes Ndc80 and Dam1 as potential interaction interfaces between these complexes, the authors now perturb these interfaces and perform optical trapping assays to probe the attachment strength between a bead coated with Ndc80 and Dam1 complexes, and dynamic microtubules. The revised version of the manuscript contains new data on the effects of Ipl1 phosphorylation on Dam1:Ndc80 coupling to the microtubule dynamics under constant force.

I congratulate the authors on an improved version of Figure 1 and accompanying description of results, which make it much clearer that Ndc80 with mutations in region A is somewhat dysfunctional. Unfortunately, this makes the experiments with Ndc80 mutant A + Dam1 complex less interesting, because it becomes difficult to disentangle the effects of Ndc80 mutant A folding, Ndc80 mutant A microtubule binding and Ndc80 mutant A : Dam1 interaction. I also note a more adequate Discussion. However, some of my comments to the initial manuscript version still remain in place after I've read the new version. I struggled particularly when reading a dense and somewhat confusing description of results on p. 6. Below are my comments to the new manuscript, which taken together prevent me from recommending this manuscript for publication.

We thank the reviewer for their additional suggestions. In retrospect, we agree that our description of results on pg. 6 was somewhat confusing, particularly because it did not properly emphasize the most important results, those obtained using Dam1 complexes phosphorylated solely at regions A, B, or C alone. With a slight rearrangement in that section of results (bottom of pg. 6), we feel the emphasis is now clearer.

1. My comment to the initial MS regarding the stoichiometry of the reported interactions is still standing. Phosphorylation of Dam1 by Ipl1 was previously reported to affect the oligomerization of Dam1, initially in Wang et al NSMB 2007, later by the authors themselves in Zelter et al Nat Comm 2015. This possibility is not discussed in the manuscript at all. Impaired Dam1 ring formation could affect the stability of bead-microtubule connection in ways similar to the ones which the authors attribute to the disruption of Dam1-Ndc80 connection (see for example, their paper by Umbreit et al. Nat Comm 2014, where presence of oligomerization-deficient Dam1 complex led to a decrease in rupture force of Ndc80/Dam1 coated beads). Coupled with incomplete Dam1 phosphorylation and unknown ratios of Ndc80 and Dam1 which apparently also vary between force ramp and force clamp assays, this complicates the correct interpretation of the authors' results.

With respect, we disagree that interpretation of our data from phosphorylated Dam1 complexes is complicated by possible effects on oligomerization. Concurrent phosphorylation of all three of the regions we studied here, A, B, and C, has little to no effect on oligomerization of the Dam1 complex, nor on its intrinsic affinity for microtubules (Gestaut 2008 *Nat Cell Biol*; Lampert 2010 *J Cell Biol*; Tien 2010 *J Cell Biol*; Zelter 2015 *Nat Commun*). The reviewer is correct that oligomerization can be inhibited by phosphorylation specifically at residue S20 on Dam1p (Zelter 2015 *Nat Commun*). (And we note that the oligomerization-deficient mutant reported in Wang 2007 *Nat Struct Mol Biol* is fully consistent with this view, because it carried a phospho-mimetic S20D substitution at this same residue.) However, in the present study, we included a phospho-blocking S20A mutation in all our phosphorylated Dam1 constructs, so that we could focus specifically on how phosphorylation affects their interaction with the

Ndc80 complex, without affecting their oligomerization. This detail is now made explicitly clear in the main text of our revised manuscript (on pg. 5). "However, oligomerization can be inhibited by phosphorylation specifically at residue S20 on Dam1p (Zelter et al., 2015). For this reason, we included a phospho-blocking S20A mutation in all our phosphorylated Dam1 constructs, so that we could specifically inhibit their interactions with the Ndc80 complex, without affecting their oligomerization."

2. In response to my comment to the initial MS regarding an almost 2-fold difference in rupture force comparing Ndc80 alone and Ndc80 + phosphorylated 6A-Dam1, the authors now report two additional sites in Dam1p (S31 and S311) as possible interaction points which were previously disregarded. However, they go on to state on p. 6 that "Phosphorylation at these residues did not affect the strength of tip-couplers". Therefore, my concern remains: the difference in rupture force between Ndc80 alone and Ndc80 + phosphorylated 6A-Dam1 indicates that there might be Ndc80-Dam1 interactions which the authors fail to disrupt by their phosphorylation treatment.

We agree that some Ndc80c-Dam1c interactions might persist even after our maximal phosphorylation treatment. (We explicitly make this point on pg. 6: "...our phosphorylation of all three regions of the Dam1 complex did not completely disrupt its load-bearing interactions with the Ndc80 complex, perhaps because we were unable to fully saturate all the phosphorylation sites of Dam1p and Ask1p.") However, this does not affect our primary conclusion that all three interacting regions, A, B, and C, participate in tip-coupling. Clear evidence that both A and B participate is shown in Fig. 1C, where substantial weakening occurs if either region A or B of Ndc80c is mutated, and also in Fig. 2B, where substantial weakening occurs if either region A or B of Dam1c is phosphorylated. Clear evidence that C participates is shown in Figs. 4 and 5, where detachments are substantially more frequent if region C is phosphorylated.

The reviewer also mentions the new phosphorylation sites we found in Dam1p, at residues S31 and S311. Our data indicate that these sites have no effect on Ndc80-Dam1 interactions, because rupture strengths using the ATP-treated phospho-deficient 6A mutant Dam1 complex remained high (8.8 pN), and indistinguishable from mock-treated Dam1 complex (8.5 pN), even though the ATP-treated 6A mutant carried high levels of phosphorylation on S31 and S311 (as indicated in the bottom row of Table 1). The rupture force distributions for these two cases overlap almost perfectly, as shown in Supplementary Fig. S5A (e.g., compare the dashed versus solid red curves in panel A), and also in a new Supplementary Fig. S4 that displays both the bead binding and rupture force data for these control experiments.

3. The authors suggest a redundancy in Dam1 regions A and B since phosphorylation in A+B is no weaker than A alone or B alone. If this were true, comparison of AB to A (n.s., Table S3) should give the same result as the comparison of AB to B ($p = 0.036$, Table S3). In other words, addition of A to B weakens the attachment, while addition of B to A does not. Therefore, my initial suggestion still stands: if the authors compare (de)phosphorylation of individual regions, like they do when describing their force clamp data, they should conclude that the only force-resistant region in Dam1 is region A. This is the only conclusion that is consistent with further comparisons: ABC to AB (n.s.), ABC to AC (n.s.), but significant difference between ABC and BC.

The reviewer's assertion, "that the only force-resistant region in Dam1 is region A", is incorrect: Figs. 2B, 4, and 5 provide clear evidence that regions B and C of the Dam1 complex both provide additional force-resistant interactions with Ndc80 complex. Rupture strength is substantially weakened by phosphorylation of B alone. (In Fig. 2B, compare red, 8.8 pN, to green, 6.8 pN; a highly significant difference, $p < 0.001$, as indicated in Table S4). Detachment rates are substantially increased by

phosphorylation of either B or C (Figs. 4 and 5; also highly significant, with $p \leq 0.004$, as indicated in Table S7).

The reviewer focuses particularly on data from multiply phosphorylated Dam1 complexes, e.g. phosphorylated at regions A and B together, or at regions B and C, or at all three regions, A, B, and C. These data are interesting, but they must be interpreted with extra caution, because the phosphorylation sites were not fully saturated (as shown in Table 1) and therefore the fraction of complexes carrying phosphorylation concurrently at every region is unclear. It is much more straightforward to interpret the data from complexes phosphorylated at individual regions, which show clearly that regions B and C contribute, and provide the basis for our primary conclusions, as explained above. We interpret the multiply phosphorylated data with caution (e.g., on pg. 6): "Phosphorylating regions A and B together caused no further weakening compared to phosphorylation of A alone, suggesting redundancy in the load-bearing function of regions A and B."

4. As I mentioned in point 3 above, the authors are inconsistent when describing the effects of Dam1 phosphorylation. In sections reporting the rupture force assays they compare phosphorylated Dam1 with alanine substitutions to phosphorylation WT Dam1. In further sections reporting the experiments at constant force phosphorylated Dam1 with alanine substitutions is compared to phosphorylated 6A-Dam1. Making these two parts consistent with each other might help the clarity of presentation.

We tested fewer combinations of phosphorylated Dam1 complexes in the constant force experiments because these experiments are far more time-consuming than the rupture force experiments. Each individual rupture force measurement requires ~30 s to complete, whereas a single constant force measurement requires many minutes, and much greater effort is required to gather statistics. We therefore focused our constant force measurements on Dam1 complexes phosphorylated only at individual regions, A, B, or C, and on the key phospho-deficient control. (These four combinations are shaded yellow in Fig. 4.) All four of these same combinations were also included in our rupture force measurements (rows 2 - 5 in Fig. 2B), so there is consistency across the two methods.

5. I do not share the authors' enthusiasm about the biological significance of the interaction region C for long-lived attachments. Rather, I think that this finding is not surprising: if the reduction in Ndc80-Dam1 affinity caused by disruption of the interaction region C is rather mild, it is quite trivial that one needs to wait longer to see the connection break. Given this explanation of the apparent discrepancy between the force-ramp and force-clamp data on region C, I do not see a reason to invoke any "interesting and important consequences for error-correction in vivo", as the authors do in the Discussion.

We disagree with the reviewer's characterization of the behavior of Dam1 complex phosphorylated at region C as "not surprising" and "quite trivial". On the contrary, it is quite surprising how differently this complex behaves relative to all the other assemblies that we have studied via both rupture force and constant force approaches: As shown in Fig. 5, the other assemblies all follow an obvious trend, with stronger rupture forces correlating with longer-lived attachments, as one might expect for a simple increase in microtubule affinity. The phospho-C Dam1 complex is a major outlier, with a far lower attachment lifetime than expected given its high rupture strength. To our knowledge, the possibility of specifically lowering the long-term stability of kinetochore-microtubule attachments while preserving their short-term strength has not previously been recognized. The behavior is especially interesting, because it can potentially explain how phosphorylation could release a mal-attached kinetochore without hampering its ability to form new, strongly load-bearing attachments – an otherwise very puzzling aspect of the prevailing models for error correction during mitosis. These points are discussed in our manuscript on pgs. 3-4 (Introduction) and pg. 9-10 (at the end of the Discussion section).

Minor point:
On p. 9 the authors write: "We demonstrate here that Ipl1p phosphorylation goes beyond regulating the affinity of the Dam1 and Ndc80 complexes for each other but also modulates the strength and stability of their interaction during tip coupling". I did not find the evidence supporting this statement in the manuscript, since the authors can not distinguish a breakage in Dam1:Ndc80 connection from breakage of Dam1-microtubule or Ndc80-microtubule connections.

Prior work established that phosphorylation of the Dam1 complex at all three regions, A, B, and C, has little to no effect on its intrinsic affinity for microtubules (Gestaut 2008 *Nat Cell Biol*; Lampert 2010 *J Cell Biol*; Tien 2010 *J Cell Biol*; Zelter 2015 *Nat Commun*) and instead strongly reduces its affinity for Ndc80 complexes on microtubules (Lampert 2010 *J Cell Biol*; Tien 2010 *J Cell Biol*; Kim 2017 *Elife*). However, as explained on pg. 4, these prior studies "did not include external force, which kinetochores must sustain almost continuously during mitosis." Thus our new study goes beyond the prior studies by establishing that phosphorylation of Dam1 at these three regions can also modulate rupture strength and attachment stability under tension.

Reviewer #2

The authors now include additional data that address how phosphorylation of three regions in Ndc80p affect the ability of NDC80C/Dam1C to maintain attachment to dynamic microtubules under load, which addresses an original major concern from this reviewer.

We are grateful to the reviewer for their careful reading of both our original and our revised manuscripts, and glad that we have successfully addressed their major concern.

I still have one remaining point that I think is worth the author's attention:

In Figure 4, the authors include data from phosphorylated complexes under constant load. The authors conclude that during both microtubule growth and shortening, all three regions contribute to maintaining attachments to microtubules under load. I would agree that the data suggest all three regions modestly contribute to maintaining attachments during microtubule growth. During shortening, it is striking that region "A" seems to be the critical domain for this function (with a possible minor contribution from C or B). One question that I still have is how the authors can rule out that the effect is at least in part due to phosphorylation of the NDC80p complex affecting its ability to remain attached to shortening microtubules directly (and not solely a consequence of loss of Dam1C assistance?). In Figure 1, this mutant seems to be 3X less likely to bind microtubules under "pre-load" than wild-type NDC80p alone. The explanation to my question may be somewhere in the text, but perhaps the authors might reiterate this point?

If we understand correctly, the reviewer is concerned that residual Ipl1 kinase and ATP, leftover from the phosphorylation reaction with Dam1 complex, might remain active enough in the trapping experiment to also phosphorylate the Ndc80 complex. This is highly unlikely, because the Dam1 complex stock, after phosphorylation, is diluted 800-fold into the final reaction mix for our constant force assays, thereby reducing the residual kinase and ATP concentrations to very low levels (0.6 nM and 12 µM, respectively), which are far below what is needed for in vitro protein phosphorylation in our experience.