

# Small changes in phospho-occupancy at the kinetochore-microtubule interface drive mitotic fidelity

Thomas Kucharski, Rufus Hards, Sarah Vandal, Maria Alba Abad, A. Arockia Jeyaparakash, Edward Kaye, Aymen al-Rawi, Tony Ly, Kristina Godek, Scott Gerber, and Duane Compton

*Corresponding Author(s): Duane Compton, Geisel School of Medicine at Dartmouth and Thomas Kucharski, Dartmouth Geisel 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.)

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September 13, 2021

Re: JCB manuscript #202107107

Dr. Duane Compton  
Geisel School of Medicine at Dartmouth  
410 remsen bldg.  
hanover, nh 03755

Dear Duane,

Thank you for submitting your manuscript entitled "Small changes in phospho-occupancy at the kinetochore-microtubule interface drive mitotic fidelity". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that opinions are mixed, with Reviewer 1 the most critical. I agree with Reviewer's 2 and 3 that the low/narrow range of phosphosite occupancy that you observe and the role of phosphatases at the kinetochore in maintaining mitotic fidelity is a conceptual advance that would be of interest to the JCB readership. However, there are a number of major technical concerns including the use of HeLa cells for most experiments that draw into question the generality of your conclusions. I direct you to the reviews for details, but the amount of work that would be necessary to satisfy the reviewers is quite substantial, and it is JCB policy to invite resubmission only if the comments can be addressed within a few months. I therefore recommend that you submit your manuscript elsewhere.

We will leave the door open, however, and if you feel that you can fully address the major criticisms of the reviewers, we would be willing to consider a revised version of your manuscript. If you would like to resubmit this work to JCB, 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, I hope that the comments below will prove constructive as your work progresses.

I am sorry that our decision is not more encouraging. I thank you again for submitting your work to JCB.

Best regards,  
Rebecca

Rebecca Heald, Ph.D.  
Editor  
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD  
Scientific Editor  
Journal of Cell Biology

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

In the manuscript entitled "Small changes in phospho-occupancy at the kinetochore-microtubule interface drive mitotic fidelity" the authors performed mass spectrometry on immunopurified Hec1 to quantify the proportion of phosphorylation sites occupied in different kinetochore attachment sites. Phospho-antibodies were prepared against some of the understudied Hec1 phosphorylation sites and were demonstrated to be enriched under conditions that promoted weak kinetochore attachments. I have some concerns regarding the manuscript which limit my enthusiasm for publication. These are summarized in the paragraphs below.

The premise for this study is well established in the mitosis field. It is known that the activity of key proteins is controlled by ongoing cycles of phosphorylation and dephosphorylation. Moreover, within the signal to noise of existing assays it is known that partial phospho-acceptor site occupancy is capable of restoring full function and that such sites are positionally promiscuous. For these reasons I find the data to be of a preliminary nature, not a significant advance in the field, and possibly over-interpreted.

It has been previously shown by the Nigg lab that changes in ploidy (analogous to the HeLa cells employed in this study) trigger changes in the phosphoproteomic response relative to euploid cells. This may artifactually influence the interpretation of Hec1

phosphorylation obtained from a HeLa lysate versus the anti-phospho antibodies visualized in RPE cells.

There is a lot of background staining in visible with the phospho-T31 antibody calling into question the specificity of the probe. If the antibodies interact with cytoplasmic Hec1, as the authors state, then this contradicts the idea that phosphorylation at T31 is specifically important for kinetochore MT attachment activity. The gels presented in Fig. S3 show that the antibodies cross-react with Hec1 and 3X-Flag-Hec1 but there is insufficient data to prove that the antibodies do not also cross-react with other components or other cdk1 substrates.

The authors state that "Given that only a modest 1.3-fold increase in phosphorylation was observed at S44 in cells treated with OA, k-MT attachments are likely very sensitive to phosphorylation levels." However, the overall sensitivity of kinetochore attachment to phosphorylation levels cannot be deduced relative to the change in one phosphorylation site (S44) using a broad spectrum phosphatase inhibitor. Especially given the effect of OA on a variety of other kinetochore-associated substrates whose contribution to attachment is uncharacterized. It might be more informative to compare these data with a more potent phosphatase inhibitor such as Calyculin A that (unlike OA) also inhibits PP1 which has been demonstrated to also stabilize end-on attachments.

The lack of a correlative effect on error correction by Hec1 T31D, while common in phosphomimic/phosphomutant studies, underscores the preliminary nature of this study. Because lagging chromosomes can arise via a wide array of mechanisms, their appearance in the phosphomutant is not unexpected but is also fundamentally mechanistically uninformative. For example, it is unknown whether T31 phosphorylation exerts a direct effect on Hec1-MT interactions or whether it influences an interaction of Hec1 with other kinetochore components which, in turn, mediate attachment.

This study implicates pT31 phosphorylation levels in the establishment of end-on kinetochore microtubule attachments. However, the authors do not convincingly distinguish between end-on, lateral and MT occupancy in their assays. Instead, the kinetochore status appears to be often inferred from previous studies. When directly recorded, the images in Fig S4e are not of sufficient resolution to distinguish a syntelic from a monotelic attachment.

In its present state the manuscript is consistent with what is already known about kinetochore phosphoregulation. However, even if the data were fleshed out, I do not think that the additional contribution of the T31 site of Hec1 to attachment defects represents a sufficient advance in understanding the regulation of kinetochore attachment, beyond that which has already been published. It has already been established that the Cdk1 pathway influences kinetochore attachment in addition to the Aurora and MPS1 phospho-regulation pathways. This manuscript endeavors to reconcile the low occupancy of phosphosites regulating Hec1 (which has been heavily studied) with the potentially greater number of potential phosphosites. The extensive published literature in this area, however, already establishes that the promiscuity of these sites operate to integrate distinct kinase/phosphatase pathways toward a similar functional end: kinetochore response to changes in KT-MT attachment.

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

The work from Kucharski et. al. is a nice characterization of the phosphorylation of the Ndc80 tail in HeLa cells. Although this is a well-studied event, it is of interest to a broad audience since it is a cornerstone of mitotic regulation. The current paper is an important extension of previous work because it applies an unbiased approach (mass spec), shows extensive characterization of a new Cdk1 site on the tail and demonstrates the importance of phosphatases in the regulation. It is surprising that Aurora and CDKs have distinct regulation on Ndc80. Overall, I found the writing clear and the conclusions interesting. I am enthusiastic about this paper as long as they strongly address my one major concern.

#### Major Concern

Almost all of the work including the CRISPR line used in the mass spectrometry is performed in HeLa cells. However, there is important work to suggest that the regulation of Aurora in response to alignment is altered in HeLa cells and other transformed cells (PMID: 21723127). I appreciate that they do demonstrate that phosphorylation on the T31 site can be detected in RPE but this is not sufficient to conclude that their conclusions are not HeLa specific. Thus, the authors risk overstating their conclusions by suggesting that they apply universally. Therefore, please perform the key experiments in a non-transformed line to confirm that they provide similar conclusions. I appreciate that it may not be trivial to repeat the mass spec but at the very least the IF with both Aurora and CDK site antibodies after Noc/STLC treatments and the phosphatase inhibitor work should be straight forward to perform in a nontransformed cell line and/or organoid system. If there are differences then this can be addressed in the writing.

#### Minor concerns

-please clearly state in the manuscript that the 3xflag was placed on the C-terminus of Hec1, I shouldn't have to spend 15 minutes of blast searching to determine this. Also please state if the flag on all copies of Hec1 or only a single allele?

-Characterization of the key cell line. Please include a western blot of WT and 3xflag cells with a Hec1 antibody so the reader can assess if the flag protein is expressed to similar levels as the endogenous protein.

-The demonstration that cyclin A2 is required to localize cyclin B2 to the spindle (both poles and kinetochores) is surprising and interesting. However there needs to be a couple of extra controls for this experiment. Please perform western blots to show:  
1) that cyclin B1 levels are similar after A2 depletion (in mitotic cells) and an IP to show that the amount of CDK1/cyclinB1 levels have not changed.  
2) that CDK1 activity in general is not depleted using a phosphoantibody. There was an antibody against a CDK site on inhibitor-2 that has been used for such quantification in the past.

-The regulation of pS31 might be a function of kinetochore microtubule attachment status as stated or a function of distance of kinetochores from poles. I may have missed the data that rules this out but if not please plot the amount of pS31 staining as a function of distance from the plane of the poles and calculate an R2 to rule out this concern.

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

The Ndc80 complex located at the outer kinetochore is essential for the formation of kinetochore-microtubule attachments. We know that both Aurora B and Cdk1 kinases phosphorylate the Ndc80 complex at consensus sites, most of which have already been established before, but the extent to which these sites are phosphorylated is not clear. Surprisingly, this work by Kucharski et al demonstrate that none of these sites attain more than 50% occupancy in any mitotic scenario, normal or abnormal. Further, there is only ~ 20% change in phospho-occupancy under different states of kinetochore-microtubule attachments. Moreover, they find that CyclinB1-Cdk1 phosphorylation (at T31 position) has a direct role in attachment error correction, similar to what has already been found for Aurora B phosphorylation of other established sites. The data from the work suggest that kinetochore phosphatase activity is responsible to maintain a highly sensitive balance of attachments within this narrow range of phospho-occupancy, while also retaining the required high fidelity of chromosome segregation. Overall, I feel this work is quite interesting and is potentially suitable for publication in JCB. I do however find several instances where there is a significant scope for improvement, which I have summarized below.

#### Major comments:

- i. One of the major problem that I am finding throughout the manuscript is that a large amount of critical data (too many instances to pinpoint) is presented as supplemental material. The way the subfigures within the main figures are organised currently, it should not be difficult to move as much of the key supplemental data to the main figures as possible.
- ii. Figure 1: Has it been characterized how much difference there exist in the kinetochore bi-attachment status between proTAME and MG132? I am also curious to know if the authors measured phospho-occupancy with Taxol treatment. Since the authors were not able to obtain the phospho-occupancy status of 4 other known sites for reasons that are relevant and understandable, how can they make completely certain correlations of the phospho-occupancy with attachment status and mitotic fidelity? I am assuming these are all Aurora B phosphorylation sites? It is probably a difficult task, but in the case of STLC treatment, would it be possible to correlate the phospho-occupancy with the attached vs unattached kinetochores by any means?
- iii. Figure 3: The authors mention that they do not obviously see Cyclin A2 at kinetochores, but why is there no Cyclin B1 kinetochore localization in Figs. 3A, 3B? Is this because antibodies were used to assess localisation? From previously published work, do these cyclins only localise to kinetochores effectively when there are exogenously expressed? Does siCyclin B1 affect the mitotic levels of Cyclin A2 by any chance? Please be clear in the figure labels of this figure and the next, whether expressed GFP-tags or antibodies are being used to assess the localisation.
- iv-a. Figure 4 and Fig. S4: I feel that the data in Fig. 3 and 4 are largely similar and could be combined. Just that two mitotic stages are shown in Fig. 3 and four stages are shown in Fig. 4. Also, I am a bit concerned that the authors are trying to make conclusive correlations about the levels of pT31, and in some cases GFP Cyclin A2/Cyclin B1 (in cell samples expressing these proteins stably or transiently?) with the status of kinetochore microtubule attachments and error correction. What is the extent of natural variations of the levels of pT31 and of these expressed proteins (compared within the same mitotic stage) observed between normal mitotic cells? I do not see any quantifications of this performed in Figs. 3 or 4. I think this analysis has to be normalized for observed natural variations before correlations can be made with results obtained in artificial conditions (like CENP-E inhibitor, OA, or treatment with other siRNAs).
- iv-b. I am not sure if the # 3 kinetochore pair in Fig. S4F is truly lateral syntelic or for that matter if the right kinetochore pair has been picked, especially if you look at the picture of the whole cell in the ACA channel above the cropped images. There seems to be a visible gap between the ACA spot and the microtubule. Please either convince me otherwise or include another example (another cell would be just fine). Also, for the # 2 monotelic, is the spindle microtubule to the attached kinetochore emanating from the opposite spindle?
- v. Figure 5: Are these experiments here performed as rescue, after KD endogenous Hec1? I think this is absolutely critical as

expression of these constructs alone could cause attachment errors. Have the authors tested for metaphase attachment errors and general loss of kinetochore microtubule stability in T31A and T31D cells? It will be good to show Fig 5C data as an inset as has been done for many other subfigures to show that the lagging chromosome does have a kinetochore in it and that it is not just a chromosome arm that is sticking out. Also, it would have been better to do the lagging chromosome analysis in RPE1 cells instead of HeLa (probably obtained from the same source of KO cells) or after Hec1 knockdown in RPE1 cells.

vi. Figure S7C: Has the strong localization of pS44 Hec1 to the spindle poles been reported before? Has similar observations been made for other phospho-specific Hec1 antibodies in other published work? Could the authors please comment upon this observation, especially in light of the observation that Cyclin B1 also localizes to this site?

vii. As a general question, apart from possible defects in error correction in prometaphase, have the authors tested if loss of pT31 levels is directly connected to defects in attachment stabilisation mechanisms in metaphase (which will also translate into lagging chromosomes in anaphase), similar to that seen with the loss of function of Cyclin A2 or B1? Does the expression or rescue with T31A Hec1 mutant lead to premature stabilisation of attachments or precocious anaphase onset?

viii. There are at least a few studies where Cdk1 has been shown to phosphorylate/control the microtubule-destabilising activity of MCAK, which in turn is critical for error correction. How can these observations be integrated with the findings from this study? Would these two mechanisms be working independently or together? What would be their relative contributions to error correction? Moreover, it might not be all that surprising that the phospho-occupancy of Hec1 is less than 50% if you consider that there are many other microtubule-binding Aurora B (and Cdk1) kinase targets at the kinetochore in addition to MCAK, such as Ska, Knl1, etc., that also contribute (directly or indirectly) to the strength of kinetochore-microtubule attachments and/or error correction. The manuscript should certainly consider/discuss these possibilities in the discussion session.

Minor comments:

i. Lines 88-91: Considering that the Ndc80 complex is the most critical target for the kinase activity that control both lateral and end-on attachments, I don't feel a need for the authors to "pick" this complex as a target to study kinetochore phospho-occupancy.

ii. Figure 2: The DAPI images here and throughout the other figures/supplemental in the manuscript look similar to blobs or masses of condensed chromosomes as observed with nocodazole treatment. Was a particular imaging condition used for acquiring images in this channel?

iii. Figure 6 & 7: Excellent set of experiments. In lines 291-93, I recommend citing Fig. 6A, 6C & S5A after the word centrosomes and citing Fig S2E at the end of the sentence as this is the only data set with microtubule staining. Looks like Fig. S3D citation is wrong here.

iv. Line 316 - detachment or attachment?

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

In the manuscript entitled "Small changes in phospho-occupancy at the kinetochore-microtubule interface drive mitotic fidelity" the authors performed mass spectrometry on immunopurified Hec1 to quantify the proportion of phosphorylation sites occupied in different kinetochore attachment sites. Phospho-antibodies were prepared against some of the understudied Hec1 phosphorylation sites and were demonstrated to be enriched under conditions that promoted weak kinetochore attachments. I have some concerns regarding the manuscript which limit my enthusiasm for publication. These are summarized in the paragraphs below.

The premise for this study is well established in the mitosis field. It is known that the activity of key proteins is controlled by ongoing cycles of phosphorylation and dephosphorylation. Moreover, within the signal to noise of existing assays it is known that partial phospho-acceptor site occupancy is capable of restoring full function and that such sites are positionally promiscuous. For these reasons I find the data to be of a preliminary nature, not a significant advance in the field, and possibly over-interpreted.

We respectfully disagree with this assessment. The measurement of phospho-occupancy of these sites has never been reported previously, and all previous results relied upon relative changes in phosphorylation. The relation of the data presented here to previous work using site-directed mutants is addressed forthrightly in our manuscript and these two different experimental approaches can't be compared one-to-one. Indeed, the previous work using site-directed mutants has led to a supposition that all the phosphorylation sites are interchangeable and our data shows that not to be the case since individual sites respond to erroneous attachments differently. Moreover, the previous work failed to include the T31 Cdk site which we show has an active role in error correction and that phosphorylation by Cdk and Aurora respond to different types of attachment errors.

It has been previously shown by the Nigg lab that changes in ploidy (analogous to the HeLa cells employed in this study) trigger changes in the phosphoproteomic response relative to euploid cells. This may artifactually influence the interpretation of Hec1 phosphorylation obtained from a HeLa lysate versus the anti-phospho antibodies visualized in RPE cells.

We thank the reviewer for their suggestion, and we concur to the outstanding scientific interest of this question. We invested substantial time to investigate this question and experienced technical challenges due to the lack of tools for synchronizing RPE1 cells with sufficient quantity and quality (cell cycle phase purity) to replicate mass spec analyses under all the conditions. However, we were able to investigate this experiment by IP/Western blot. This experiment showed essentially no difference between HeLa and RPE1 cells in phosphorylating Hec1 on T31 on the same quantity of total Hec1. This experiment is now presented as figure 2C. We also have used quantitative immunofluorescence analysis to compare the intensity of phospho-Hec1 relative to total Hec1 in these two cell lines under the various conditions, and that also shows comparable phosphorylation.

There is a lot of background staining in visible with the phospho-T31 antibody calling into question the specificity of the probe. If the antibodies interact with cytoplasmic Hec1, as the authors state, then this contradicts the idea that phosphorylation at T31 is specifically important for kinetochore MT attachment activity. The gels presented in Fig. S3 show that the antibodies cross-react with Hec1 and 3X-Flag-Hec1 but there is insufficient data to prove that the antibodies do not also cross-react with other components or other cdk1 substrates.

We respectfully disagree with this assessment. We specifically show in figure S3A that the anti pT31 antibody only recognizes phosphorylated kinetochores containing a serine at location 31. We admit that some background staining is visible, but some of that is inherent to phospho-antibodies and the literature is replete with such examples. That is why we always measure kinetochore fluorescence and not whole-cell fluorescence.

The authors state that "Given that only a modest 1.3-fold increase in phosphorylation was observed at S44 in cells treated with OA, k-MT attachments are likely very sensitive to phosphorylation levels." However, the overall sensitivity of kinetochore attachment to phosphorylation levels cannot be deduced relative to the change in one phosphorylation site (S44) using a broad spectrum phosphatase inhibitor. Especially given the effect of OA on a variety of other kinetochore-associated substrates whose contribution to attachment is uncharacterized. It might be more informative to compare these data with a more potent phosphatase inhibitor such as Calyculin A that (unlike OA) also inhibits PP1 which has been demonstrated to also stabilize end-on attachments.

We thank the reviewer for their suggestion. This data is now shown as figure S6. Interestingly, Calyculin A did not affect Hec1 (both pS44 and pT31) as strongly as Okadaic acid, and accordingly did not cause kinetochore detachment from microtubules. However, we note that this might have been because of extremely high toxicity at higher doses which limited our analysis to a dose of only 5 nM.

The lack of a correlative effect on error correction by Hec1 T31D, while common in phosphomimic/phosphomutant studies, underscores the preliminary nature of this study. Because lagging chromosomes can arise via a wide array of mechanisms, their appearance in the phosphomutant is not unexpected but is also fundamentally mechanistically uninformative. For example, it is unknown whether T31 phosphorylation exerts a direct effect on Hec1-MT interactions or whether it influences an interaction of Hec1 with other kinetochore components which, in turn, mediate attachment.

We thank the reviewer for their very interesting suggestion. We note that there is substantial published work by many labs showing a direct source of lagging chromosomes resulting from persistent errors in kinetochore-microtubule attachment. Since expression of T31A causes a large increase in the number of lagging chromosomes, we believe that we can justifiably claim that T31 and therefore Cdk1-CycB1 is important for error correction.

Nevertheless, the reviewer's suggestion raises a technically challenging question that would require a separate effort to evaluate binding events using in vitro biochemical assays. That is clearly beyond the scope of the current work, in which, we are already receiving concerns about the length and current scope.

This study implicates pT31 phosphorylation levels in the establishment of end-on kinetochore microtubule attachments. However, the authors do not convincingly distinguish between end-on, lateral and MT occupancy in their assays. Instead, the kinetochore status appears to be often inferred from previous studies. When directly recorded, the images in Fig S4e are not of sufficient resolution to distinguish a syntelic from a monotelic attachment.

We thank the reviewer for their suggestion. Upon reassessment of the data, we agree that the images in figure S4 are of insufficient resolution to distinguish between monotelic and syntelic attachment configurations. However, they are sufficient to distinguish end-on from lateral. Therefore, we have adjusted the figure and text to reflect that and to be more conservative in our claims.

In its present state the manuscript is consistent with what is already known about kinetochore phosphoregulation. However, even if the data were fleshed out, I do not think that the additional contribution of the T31 site of Hec1 to attachment defects represents a sufficient advance in understanding the regulation of kinetochore attachment, beyond that which has already been published. It has already been established that the Cdk1 pathway influences kinetochore attachment in addition to the Aurora and MPS1 phospho-regulation pathways. This manuscript endeavors to reconcile the low occupancy of phosphosites regulating Hec1 (which has been heavily studied) with the potentially greater number of potential phosphosites. The extensive published literature in this area, however, already

establishes that the promiscuity of these sites operate to integrate distinct kinase/phosphatase pathways toward a similar functional end: kinetochore response to changes in KT-MT attachment.

We discovered a new role for Cdk1-Cyclin B1 in error correction, determine the absolute phospho-occupancy of an important mitotic regulator, discovered and describe the dynamics of a new and unexpected phosphorylation site on Hec1, describe a valuable new reagent for studying cyclin B1 activity at the kinetochore (anti pT31 antibody), and show that Cdk and Aurora respond to different attachment errors. We feel that these contributions provide sufficient new mechanistic insight to justify publication in JCB.

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

The work from Kucharski et. al. is a nice characterization of the phosphorylation of the Ndc80 tail in HeLa cells. Although this is a well-studied event, it is of interest to a broad audience since it is a cornerstone of mitotic regulation. The current paper is an important extension of previous work because it applies an unbiased approach (mass spec), shows extensive characterization of a new Cdk1 site on the tail and demonstrates the importance of phosphatases in the regulation. It is surprising that Aurora and CDKs have distinct regulation on Ndc80. Overall, I found the writing clear and the conclusions interesting. I am enthusiastic about this paper as long as they strongly address my one major concern.

**Major Concern**

Almost all of the work including the CRISPR line used in the mass spectrometry is performed in HeLa cells. However, there is important work to suggest that the regulation of Aurora in response to alignment is altered in HeLa cells and other transformed cells (PMID: 21723127). I appreciate that they do demonstrate that phosphorylation on the T31 site can be detected in RPE but this is not sufficient to conclude that their conclusions are not HeLa specific. Thus, the authors risk overstating their conclusions by suggesting that they apply universally. Therefore, please perform the key experiments in a non-transformed line to confirm that they provide similar conclusions. I appreciate that it may not be trivial to repeat the mass spec but at the very least the IF with both Aurora and CDK site antibodies after Noc/STLC treatments and the phosphatase inhibitor work should be straight forward to perform in a nontransformed cell line and/or organoid system. If there are differences then this can be addressed in the writing.

We thank the reviewer for their suggestion. This concern was also raised by reviewer #1, and we agree that this question is of outstanding scientific interest. We refer the reviewer to our response to reviewer #1.

**Minor concerns**

-please clearly state in the manuscript that the 3xflag was placed on the C-terminus of Hec1, I shouldn't have to spend 15 minutes of blast searching to determine this. Also please state if the flag on all copies of Hec1 or only a single allele?

We regret the trouble for the reviewer. However, we always refer to the protein as Hec1-3XFLAG, which implies c-terminal localization, as opposed to 3XFLAG-Hec1. We mentioned on line 3 of the results section that the modification was heterozygous. We also provided a cartoon depicting the modifications to the Hec1 gene as figure S1A, and genotyping as S1B.

-Characterization of the key cell line. Please include a western blot of WT and 3xflag cells with a Hec1 antibody so the reader can assess if the flag protein is expressed to similar levels as the endogenous protein.

We thank the reviewer for their suggestion. We note that this was included in the manuscript in figure S2A.

-The demonstration that cyclin A2 is required to localize cyclin B2 to the spindle (both poles and kinetochores) is surprising and interesting. However there needs to be a couple of extra controls for this experiment. Please perform western blots to show:  
1) that cyclin B1 levels are similar after A2 depletion (in mitotic cells) and an IP to show that the amount of CDK1/cyclinB1 levels have not changed.  
2) that CDK1 activity in general is not depleted using a phosphoantibody. There was an antibody against a CDK site on inhibitor-2 that has been used for such quantification in the past.

We thank the reviewer for their valuable suggestion. This was an oversight on our part and has now been corrected. We opted to use lamin A/C pS22 (a well characterized Cdk1-CycB site) for a functional demonstration that silencing CycA2 does not affect CycB1 function outside of the kinetochore. We clearly show that CycB1 knockdown and not CycA2 knockdown significantly affect levels of lamin A/C pS22. Since levels of lamin A/C pS22 are not affected, this also implies that levels of Cyclin B1 are not affected.

-The regulation of pS31 might be a function of kinetochore microtubule attachment status as stated or a function of distance of kinetochores from poles. I may have missed the data that rules this out but if not please plot the amount of pS31 staining as a function of distance from the plane of the poles and calculate an R2 to rule out this concern.

We thank the reviewer for their valuable suggestion. We have now provided this data as Figure 5 B and C. No correlation between pT31 levels and kinetochore-to-pole distance was apparent.

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

The Ndc80 complex located at the outer kinetochore is essential for the formation of kinetochore-microtubule attachments. We know that both Aurora B and Cdk1 kinases phosphorylate the Ndc80 complex at consensus sites, most of which have already been established before, but the extent to which these sites are phosphorylated is not clear. Surprisingly, this work by Kucharski et al demonstrate that none of these sites attain more than 50% occupancy in any mitotic scenario, normal or abnormal. Further, there is only ~ 20% change in phospho-occupancy under different states of kinetochore-microtubule attachments. Moreover, they find that CyclinB1-Cdk1 phosphorylation (at T31 position) has a direct role in attachment error correction, similar to what has already been found for Aurora B phosphorylation of other established sites. The data from the work suggest that kinetochore phosphatase activity is responsible to maintain a highly sensitive balance of attachments within this narrow range of phospho-occupancy, while also retaining the required high fidelity of chromosome segregation. Overall, I feel this work is quite interesting and is potentially suitable for publication in JCB. I do however find several instances where there is a significant scope for improvement, which I have summarized below.

**Major comments:**

i. One of the major problem that I am finding throughout the manuscript is that a large amount of critical data (too many instances to pinpoint) is presented as supplemental material. The way the subfigures within the main figures are organised currently, it should not be difficult to move as much of the key supplemental data to the main figures as possible.

We thank the reviewer for their suggestion. We have taken great care to provide a clear and logical flow to the text and the figures.

ii. Figure 1: Has it been characterized how much difference there exist in the kinetochore bi-attachment status between proTAME and MG132? I am also curious to know if the authors measured phospho-occupancy with Taxol treatment. Since the authors were not able to obtain the phospho-occupancy status of 4 other known sites for reasons that are relevant and understandable, how can they make completely certain correlations of the phospho-occupancy with attachment status and mitotic fidelity? I am assuming these are all Aurora B phosphorylation sites? It is probably a difficult task, but in the case of STLC treatment, would it be possible to correlate the phospho-occupancy with the attached vs unattached kinetochores by any means?

We thank the reviewer for their suggestion and interesting comments. To our knowledge, the differences between proTAME and MG132 have not been determined. We prefer proTAME for two important reasons: MG132 blocks mitotic entry because it prevents wee1 degradation by the SCF/proteasome just prior to mitosis. This causes levels of the inhibitory phosphorylation on Cdk1 to remain elevated, preventing Cdk1 activation and mitotic entry. Secondly, proTAME does not prevent cyclin A2 degradation (which is important for K-MT stability), thus providing a more faithful metaphase model.

We did not test the effect of taxol in our system. We prefer to avoid it due to the artificial stabilization of K-MT attachments, which has no parallel in normal mitosis.

Determining the absolute phospho-occupancy of attached vs unattached kinetochores would be of very high interest. However, to do so would mean using the numbers from the MS analysis, comparing them to the IF, images, and then deconvoluting them with respect to attached/unattached kinetochores. We feel that this analysis would introduce an unacceptable level of error. However, if it becomes technically possible, we would be very happy to determine this in the future. Notably, we did provide MS data from cells treated with a high dose of nocodazole which completely prevents K-MT attachment and showed approximately 20% occupancy.

iii. Figure 3: The authors mention that they do not obviously see Cyclin A2 at kinetochores, but why is there no Cyclin B1 kinetochore localization in Figs. 3A, 3B? Is this because antibodies were used to assess localisation? From previously published work, do these cyclins only localise to kinetochores effectively when there are exogenously expressed? Does siCyclin B1 affect the mitotic levels of Cyclin A2 by any chance? Please be clear in the figure labels of this figure and the next, whether expressed GFP-tags or antibodies are being used to assess the localisation.

The reviewer is correct, the antibodies used for this analysis are very inefficient for IF. They do show overall levels acceptably, but we do not trust them for fine analysis. We do not have any reason to believe that siCyclin B1 affects Cyclin A2 levels. We have made adjustments to improve the clarity. GFP fusions are always labelled as such and the stained proteins are always stated in the figure legends.

iv-a. Figure 4 and Fig. S4: I feel that the data in Fig. 3 and 4 are largely similar and could be combined. Just that two mitotic stages are shown in Fig. 3 and four stages are shown in Fig. 4. Also, I am a bit concerned that the authors are trying to make conclusive correlations about the levels of pT31, and in some cases GFP Cyclin A2/Cyclin B1 (in cell samples expressing these proteins stably or transiently?) with the status of kinetochore microtubule attachments and error correction. What is the extent of natural variations of the levels of pT31 and of these expressed proteins (compared within the same mitotic stage) observed between normal mitotic cells? I do not see any quantifications of this performed in Figs. 3 or 4. I think this analysis has to be normalized for observed natural variations before correlations can be made with results obtained in artificial conditions (like CENP-E inhibitor, OA, or treatment with other siRNAs).

We thank the reviewer for their suggestion. It is true that the figures are related, but for spacing and sizing reasons and, added data for the revised version we have opted to leave them separate.

The GFP-fusion proteins are stably expressed. As we have shown, in Figure 5 and others, there is significant variation in pT31 levels, ranging from undetectable to robust, which correlates with the attachment status of the kinetochore. We did not provide quantification for these data for two reasons: Firstly, antibody species usage prevented normalizing to total Hec1 levels, which we feel is extremely important (and something that few other publications have done). Secondly, the effects are so profound that no quantification is required, there is no pT31 signal in cells with siCycA2 or siCycB1. Similarly, there is no CycB1 at kinetochores with siCycA2.

iv-b. I am not sure if the # 3 kinetochore pair in Fig. S4F is truly lateral syntelic or for that matter if the right kinetochore pair has been picked, especially if you look at the picture of the whole cell in the ACA channel above the cropped images. There seems to be a visible gap between the ACA spot and the microtubule. Please either convince me otherwise or include another example (another cell would be just fine). Also, for the # 2 monotelic, is the spindle microtubule to the attached kinetochore emanating from the opposite spindle?

We thank the reviewer for their suggestion. This concern was also raised by reviewer #1, and we refer the reviewer to our response to reviewer #1.

v. Figure 5: Are these experiments here performed as rescue, after KD endogenous Hec1? I think this is absolutely critical as expression of these constructs alone could cause attachment errors. Have the authors tested for metaphase attachment errors and general loss of kinetochore microtubule stability in T31A and T31D cells? It will be good to show Fig 5C data as an inset as has been done for many other subfigures to show that the lagging chromosome does have a kinetochore in it and that it is not just a chromosome arm that is sticking out. Also, it would have been better to do the lagging chromosome analysis in RPE1 cells instead of HeLa (probably obtained from the same source of KO cells) or after Hec1 knockdown in RPE1 cells.

Yes, these experiments are performed as rescue, after endogenous Hec1 knockout. We have edited the text and figures for improved clarity.

We thank the reviewer for their suggestion. Studying K-MT stability in cells expressing these mutants was also raised by reviewer #1. We refer the reviewer to our response to reviewer #1.

We have provided the requested insets.

We thank the reviewer for their suggestion. We feel that studying this question in RPE1 cells would be very difficult because of their inherently low lagging chromosome rate.

vi. Figure S7C: Has the strong localization of pS44 Hec1 to the spindle poles been reported before? Has similar observations been made for other phospho-specific Hec1 antibodies in other published work? Could the authors please comment upon this observation, especially in light of the observation that Cyclin B1 also localizes to this site?

We thank the reviewer for raising this point. In our experience, almost every antibody we have used against an Aurora substrate cross-reacts with the spindle poles due to the abundance of aurora sites there. This signal is erroneous and not from Hec1. Similar staining is visible in other studies (e.g. DeLuca et al. 2011).

vii. As a general question, apart from possible defects in error correction in prometaphase, have the authors tested if loss of pT31 levels is directly connected to defects in attachment stabilisation mechanisms in metaphase (which will also translate into lagging chromosomes in anaphase), similar to

that seen with the loss of function of Cyclin A2 or B1? Does the expression or rescue with T31A Hec1 mutant lead to premature stabilisation of attachments or precocious anaphase onset?

We thank the reviewer for raising this interesting point. As noted above, we have not investigated if loss of pT31 is directly connected to defects in attachment stabilization. However, using the lagging chromosome rate as a readout for attachment stability, we believe that expression of this mutant raises K-MT attachment stability.

viii. There are at least a few studies where Cdk1 has been shown to phosphorylate/control the microtubule-destabilising activity of MCAK, which in turn is critical for error correction. How can these observations be integrated with the findings from this study? Would these two mechanisms be working independently or together? What would be their relative contributions to error correction? Moreover, it might not be all that surprising that the phospho-occupancy of Hec1 is less than 50% if you consider that there are many other microtubule-binding Aurora B (and Cdk1) kinase targets at the kinetochore in addition to MCAK, such as Ska, Knl1, etc., that also contribute (directly or indirectly) to the strength of kinetochore-microtubule attachments and/or error correction. The manuscript should certainly consider/discuss these possibilities in the discussion session.

We thank the reviewer for raising this interesting point. This current work has been focused on Hec1 because of its role in the kinetochore-microtubule binding interface. Expansion of this effort to consider the role of CDK phosphorylation of MCAK (or other kinetochore proteins) is something that we are excited to do although that is clearly beyond the scope of the current work. At this time, it would be overly speculative for us to propose the relative contributions of Hec1 and MCAK through phosphorylation of Cdk.

Minor comments:

i. Lines 88-91: Considering that the Ndc80 complex is the most critical target for the kinase activity that control both lateral and end-on attachments, I don't feel a need for the authors to "pick" this complex as a target to study kinetochore phospho-occupancy.

We thank the reviewer for noting the importance of this protein complex.

ii. Figure 2: The DAPI images here and throughout the other figures/supplemental in the manuscript look similar to blobs or masses of condensed chromosomes as observed with nocodazole treatment. Was a particular imaging condition used for acquiring images in this channel?

We found that the best staining conditions for the pT31 antibody used cold methanol for cell fixation. However, methanol is not compatible with GFP fluorescence, so we had to use formaldehyde for experiments involving GFP-fusion proteins. Formaldehyde is far superior to methanol for DAPI signal quality. Also, we deconvolved all images for presentation and analysis, which was not optimal for the DAPI signal from methanol fixed cells.

iii. Figure 6 & 7: Excellent set of experiments. In lines 291-93, I recommend citing Fig. 6A, 6C & S5A after the word centrosomes and citing Fig S2E at the end of the sentence as this is the only data set with microtubule staining. Looks like Fig. S3D citation is wrong here.

We thank the reviewer for their suggestion and have fixed the figure references.

iv. Line 316 - detachment or attachment?

We thank the reviewer for their suggestion and have fixed this sentence.

May 27, 2022

Re: JCB manuscript #202107107R-A

Dr. Duane Compton  
Geisel School of Medicine at Dartmouth  
410 remsen bldg.  
hanover, nh 03755

Dear Duane,

Thank you for submitting your revised manuscript entitled "Chromosome segregation fidelity is controlled by small changes in phospho-occupancy at the kinetochore-microtubule interface". The manuscript has been seen by the original reviewers whose full comments are appended below. We apologize for the delay in communicating our decision to you. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that there are still a few points to address before we can move ahead with publication. Reviewer 1 would like you to re-frame your findings and model in the context of published work describing partial occupancy of phosphorylation sites. Reviewer 3 still requests a more definitive experiment to support the connection between Hec1(T31) phosphorylation and kinetochore microtubule stability. If you can address these and the other remaining concerns of the reviewers, we would be willing to consider one last version of your manuscript.

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

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

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu).

Sincerely,  
Rebecca

Rebecca Heald, Ph.D.  
Editor  
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD  
Scientific Editor  
Journal of Cell Biology

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

The authors state that phosphorylation occurs as an all-or-none phenomenon. This appears to misinterpret the data from Olsen et al. (2010) in which the model for occupancy was derived from singly phosphorylated peptides. The present manuscript investigates the multiple occupancy of one peptide (Hec1) which is not contradictory to this model. Furthermore, it is consistent with the average occupancy of phosphorylated motifs in other large scale investigations (Rao and Moller 2011; Carpy et al. 2014). Those studies report, similar to this study, that greater than 50% occupancy is rare. A modest increase was noted by Carpy et al. in mitotic cells. Although the latter study was performed in *S. pombe*, Ndc80 is a highly conserved protein. Furthermore, the occupancy of phosphorylation motifs is thought to be strongly dependent on the distance between the sites (sites closer than 10 amino acids) are thought to be less likely than fully occupied due to repulsion between the phosphate groups (Rao and Moller, 2011). Thus the data presented do not appear to be in context with the existing published data and I strongly disagree that these data overturn an established model for regulation by phosphorylation. I also disagree that the partial occupancy of phospho-sites within Hec1 is reflective of functionality because partial occupancy is the rule rather than the exception in the literature for a vast number of phosphoproteins. Instead, partial occupancy may reflect the chemistry of the reaction. I believe the authors need to re-write their partial occupancy data to reflect published findings that partial occupancy is consistent with other phospho-screens in favor of emphasizing the potential role of the T31 site in Hec1. Accordingly, the

phosphorylation state of the T31-P site might be required for Ska docking (cdk1 is a known regulator of Ska-ndc80 association) rather than MT release.

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

The authors have addressed my concerns in a convincing manner and I now support publication.

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

The manuscript is much improved and the authors have addressed most of my questions. However they have not satisfactorily addressed two remaining concerns from my previous review:

The 1st is related to the natural variations of the levels of pT31 and of the expressed proteins observed in normal mitotic cells within the same mitotic stage(s). I would like to see an effort made towards performing at least some of these quantifications before a deduction is made regarding the possibility that too much error will be introduced in these measurements. I think this is critical to make sense of some of their data and to enable comparisons with some of the artificial conditions such as treatment with CENP-E or phosphatase inhibitors.

I am also not satisfied by the response concerning the testing of metaphase attachment errors and about testing for general loss of kinetochore microtubule stability in T31A and T31D cells. This question has come-up twice in my review and the authors have failed to address this adequately. This can be addressed by a simple cold-treatment or FRAP experiments in cells expressing photoactivatable GFP-tubulin and I think that at least one of these experiments should be attempted.

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

The authors state that phosphorylation occurs as an all-or-none phenomenon. This appears to misinterpret the data from Olsen et al. (2010) in which the model for occupancy was derived from singly phosphorylated peptides. The present manuscript investigates the multiple occupancy of one peptide (Hec1) which is not contradictory to this model. Furthermore, it is consistent with the average occupancy of phosphorylated motifs in other large scale investigations (Rao and Moller 2011; Carpy et al. 2014). Those studies report, similar to this study, that greater than 50% occupancy is rare. A modest increase was noted by Carpy et al. in mitotic cells. Although the latter study was performed in *S. pombe*, Ndc80 is a highly conserved protein. Furthermore, the occupancy of phosphorylation motifs is thought to be strongly dependent on the distance between the sites (sites closer than 10 amino acids) are thought to be less likely than fully occupied due to repulsion between the phosphate groups (Rao and Moller, 2011). Thus the data presented do not appear to be in context with the existing published data and I strongly disagree that these data overturn an established model for regulation by phosphorylation. I also disagree that the partial occupancy of phospho-sites within Hec1 is reflective of functionality because partial occupancy is the rule rather than the exception in the literature for a vast number of phosphoproteins. Instead, partial occupancy may reflect the chemistry of the reaction. I believe the authors need to re-write their partial occupancy data to reflect published findings that partial occupancy is consistent with other phospho-screens in favor of emphasizing the potential role of the T31 site in Hec1. Accordingly, the phosphorylation state of the T31-P site might be required for Ska docking (cdk1 is a known regulator of Ska-ndc80 association) rather than MT release.

We thank the reviewer for their suggestions on the framing of our work. Especially for highlighting two studies that we were unaware of. In the phosphoproteomic studies they mention, Hec1 (NDC80 in yeast) does not appear. However, it does appear once in the paper by Olsen et al., as 63% occupied on an ambiguously doubly phosphorylated peptide (pS69/72). We include this information in the results section of the revised manuscript.

We also draw greater attention to the previous findings in the discussion section of the manuscript and use two of the peptides from their results as contrasting situations. One is the highly phosphorylated lamin A pS22, and the other is moderately phosphorylated PLK1 pT210. We suggest that high occupancy is used in cases where it is desirable for a strong and unidirectional effect on protein function, whereas moderate phosphorylation occupancy is used in situations where up or down regulation is required in the context of cellular feedback loops.

The information provided in the Rao & Moller 2012 paper is very interesting, and we agree that steric repulsion between phosphates plays an important role on setting occupancy levels at closely-spaced phosphoacceptors sites. We now explicitly cite that work in the discussion section of the manuscript as part of a mechanism to dampen phosphorylation occupancy and we elaborate on how it would apply to our model for the regulation of Hec1.

The reviewer also mentions the possibility that phosphorylation of Hec1 at T31 might have functions other than the regulation of K-MT stability, such as Ska complex recruitment. While we of course cannot rule out other functions which have not yet been discovered, we did test if mutation of T31 to A or D affects Ska recruitment, using the Ska3 subunit as a marker for the complex. We found that mutation of T31 to A or D did not affect Ska3 recruitment to the kinetochore in metaphase cells. This data is now presented as figure S5C and D.

Moreover, we also carried out the experiment on cold-stable microtubules as suggested by reviewer #3. This experiment revealed a statistically significant increase in cold-stable microtubule content in cells expressing the T31A Hec1 mutant, as well as in cells expressing the 9A mutant that we use as a positive control in this assay. Taken together, we believe that these two experiments draw a strong case that Hec1 T31 phosphorylation is involved in setting K-MT attachment stability and not in other cellular processes. This data is now presented as figure 5 I and J.

We thank this reviewer for their thoughtful suggestions and we believe that we have adequately addressed all of their concerns.

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

The authors have addressed my concerns in a convincing manner and I now support publication.

We thank the reviewer for their support.

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

The manuscript is much improved and the authors have addressed most of my questions. However they have not satisfactorily addressed two remaining concerns from my previous review:

The 1st is related to the natural variations of the levels of pT31 and of the expressed proteins observed in normal mitotic cells within the same mitotic stage(s). I would like to see an effort made towards performing at least some of these quantifications before a deduction is made regarding the possibility that too much error will be introduced in these measurements. I think this is critical to make sense of some of their data and to enable comparisons with some of the artificial conditions such as treatment with CENP-E or phosphatase inhibitors.

We thank the reviewer for highlighting this potential issue. We are aware that Hec1 levels vary at the kinetochore during mitosis as shown previously (Magidson et al. NCB 2015) which is why we normalized staining intensity of phosphor-epitopes to total Hec1 in all of our experiments. Perhaps, the reviewer did not recognize that we were doing so.

Nevertheless, we recognize from the comment raised by this reviewer, that it would be valuable for us to more explicitly call out that fact. Thus, we have now provided the data for the levels of total Hec1 protein (i.e. non-phospho) at the kinetochore in our assays (Figure 2C, S3 H,K, and others). These data show elevated levels of Hec1 at erroneously attached kinetochores, as well as in prometaphase compared to prophase, metaphase and anaphase. This highlights the importance of normalizing phospho-signal to the total protein signal, which we provide in all assays where it is measured.

Furthermore, to better illustrate the natural variation in Hec1 levels, we have revised the presentation of our data as dot plots (where applicable) showing the full distribution of the data, which fits with statistical trends in the field. The actual data and values reported are unchanged compared to the previous manuscript versions.

Importantly, the dot plot figure 2B that is calibrated to the mass spectrometry data fits very nicely in a scale of 0-100% absolute occupancy, notwithstanding a few outlier points that are to be expected. Thus, in prophase T31 is phosphorylated at most kinetochores from 0-20%, prometaphase ~20-70%, metaphase ~10-40% and anaphase ~0-20%. However, the distribution of data clearly shows that cells do use the full scale of potential occupancy in mitosis, which makes sense considering the changes in K-MT attachments during mitotic progression that are already known, i.e. no attachments in prophase, then a heterogeneous mix of erroneous and end-on attachments early in mitosis moving towards 100% end-on attachments at metaphase.

I am also not satisfied by the response concerning the testing of metaphase attachment errors and about testing for general loss of kinetochore microtubule stability in T31A and T31D cells. This question has come-up twice in my review and the authors have failed to address this adequately. This can be addressed by a simple cold-treatment or FRAP experiments in cells expressing photoactivatable GFP-tubulin and I think that at least one of these experiments should be attempted.

We thank the reviewer for their suggestion. We fully agree that the experiment significantly strengthens our conclusions that Hec1 T31 phosphorylation plays a role in setting K-MT attachment stability. We have executed the cold-stable assay as suggested by the reviewer since it was more feasible considering the timeline for revisions than photoactivation. This experiment revealed a statistically significant increase in cold-stable microtubule content in cells expressing the T31A Hec1 mutant, as well as in cells expressing the 9A mutant that we use as a positive control in this assay. These and other data presented in this manuscript draw a strong case that phosphorylation of Hec1 T31 by Cdk-Cyclin B1 directly participates in setting K-MT attachment stability to facilitate correction of erroneous K-MT attachments to support chromosome segregation fidelity. This data is now presented as figure 5I and J.

June 22, 2022

RE: JCB Manuscript #202107107RR

Dr. Duane Compton  
Geisel School of Medicine at Dartmouth  
410 remsen bldg.  
hanover, nh 03755

Dear Duane:

Thank you for submitting your revised manuscript entitled "Small changes in phospho-occupancy at the kinetochore-microtubule interface drive mitotic fidelity". We have now assessed your revised manuscript and would be happy to publish your paper in JCB pending revisions 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. Please go through all the formatting points paying special attention to those marked with asterisks.

#### A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, <https://jcb.rupress.org/submission-guidelines#revised>.

**\*\*Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.\*\***

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

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

3) Figure formatting:

Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

**\*\*\* Scale bars must be present on all microscopy images, including inset magnifications. Please, include scale bars in main Figs. 3D, 5F (inset magnifications).**

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

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. Please, also indicate whether N refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). We are aware that you have provided this information in the Methods, but we would need that you also include it in the figure legends where appropriate.**

Statistical methods should be explained in full in the materials and methods.

For figures presenting pooled data the statistical measure should be defined in the figure legends.

**\*\*\* Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). We are aware that you have provided this information in the Methods, but we would need that you also include it in the figure legends where appropriate.**

**\*\*\* As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally**

distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, 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 last sentence of the abstract appears a bit confusing, thus we would like to suggest that you consider rewording it to improve clarity.

The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods:

\*\*\* Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials. Please, provide full description of 'SDS-PAGE and Western blot'.

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 include species for all of your antibodies.

8) Microscope image acquisition:

The following information must be provided about the acquisition and processing of images:

- a. Make and model of microscope
- b. Type, magnification, and numerical aperture of the objective lenses
- c. Temperature
- d. imaging medium
- e. Fluorochromes
- f. Camera make and model
- g. Acquisition software
- h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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

10) Supplemental materials:

\*\*\* There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables. Currently, you have 8 supplemental figures. We could give you a bit of extra space, but we would need that you reduce the number of supplemental figures -you may consider combining two supplemental figures or move some of them to main figures (you can have up to 10 main figures).

\*\*\* A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

\*\*\* Please note that supplemental figures and tables should be provided as individual, editable files.

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. It should begin with "First author name(s) et al..." to match our preferred style.

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) A separate author contribution section is required following the Acknowledgments in all research manuscripts.

\*\*\* All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature should be used (<https://casrai.org/credit/>).

14) 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.

15) Materials and data sharing:

As a condition of publication, authors must make protocols and unique materials (including, but not limited to, cloned DNAs; antibodies; bacterial, animal, or plant cells; and viruses) described in our published articles freely available upon request by researchers, who may use them in their own laboratory only. All materials must be made available on request and without undue delay. Please, indicate whether the materials generated in this study have been deposited in public repositories. If not, please state that they would be made available to the scientific community upon request in the 'Data availability' section.

\*\*\* All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: <https://rupress.org/jcb/pages/data-deposition>), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database. Please, deposit your mass spectrometry data in a proper public repository and include the accession number in the Methods.

16) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. The Source Data files will be directly linked to specific figures in the published article.

\*\*\* Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

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

## B. FINAL FILES:

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](mailto:lhollander@rockefeller.edu)).

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

-- High-resolution figure and MP4 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.\*\*

\*\*The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.\*\*

Additionally, JCB encourages authors to submit a short video summary of their work. These videos are intended to convey the main messages of the study to a non-specialist, scientific audience. Think of them as an extended version of your abstract, or a short poster presentation. We encourage first authors to present the results to increase their visibility. The videos will be shared on social media to promote your work. For more detailed guidelines and tips on preparing your video, please visit <https://rupress.org/jcb/pages/submission-guidelines#videoSummaries>.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. Please let us know if any complication preventing you from meeting this deadline arises and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, [cellbio@rockefeller.edu](mailto:cellbio@rockefeller.edu).

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

Sincerely,  
Rebecca

Rebecca Heald, Ph.D.  
Editor  
The Journal of Cell Biology

Lucia Morgado-Palacin, PhD  
Scientific Editor  
Journal of Cell Biology

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