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Nuclear-enriched protein phosphatase 4 ensures outer kinetochore assembly before nuclear dissolution

Helder Rocha, Patrícia Simões, Jacqueline Budrewicz, Pablo Lara-Gonzalez, Ana Xavier Carvalho, Julien Dumont, Arshad Desai, and Reto Gassmann

Corresponding Author(s): Reto Gassmann, Institute for Molecular and Cell Biology

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October 19, 2022

Re: JCB manuscript #202208154

Dr. Reto Gassmann
Institute for Molecular and Cell Biology
Rua Alfredo Allen, 208
Porto, Porto 4200-135
Portugal

Dear Dr. Gassmann,

Thank you for submitting your manuscript entitled "Nuclear-enriched protein phosphatase 4 ensures outer kinetochore assembly before nuclear dissolution." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that while Reviewer #1 expresses reservations about the degree of conceptual advance, the other two reviewers feel the study provides interesting and important findings. We agree that your study is suitable for the JCB Report format, which would not require a full investigation of how PP4 functions in outer kinetochore assembly. However, please make sure to address all other comments with additional data where requested.

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

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

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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

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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

The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision

cycle, so any revised manuscript will likely be either accepted or rejected.

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

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

Daniela Cimini, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In this study, the authors investigate the role of the PP4 phosphatase in mitotic chromosome segregation in C. elegans. There is much to learn about this particular phosphatase family, as its function in mitosis has been relatively understudied, especially in comparison to the PP1 and PP2A phosphatase families.

Here the authors identified SMK-1, a PP4 regulatory subunit, in a screen for proteins involved in early embryonic mitosis in C. elegans. Depletion of SMK-1 results in a striking mitotic phenotype: chromosomes are initially scattered, but after a delay they eventually align and form kinetochore-microtubule attachments that are capable of generating tension. Cells go on to divide, although they do so with significant chromosome segregation errors. The authors go on to show that PP4 is required for timely assembly of the outer kinetochore, and they specifically show that KNL1 and HIM-10/Nuf2 (of the Ndc80 complex), as well as Spindly and dynein experience delayed recruitment to kinetochores. After ruling out several other mechanisms, they conclude that the chromosome scattering defect is a result of delayed formation of kinetochore-microtubule attachments (due to delayed formation of the outer kinetochore). They also demonstrate that the outer kinetochore NDC80C-SkaC 'module' is required for chromosome alignment recovery after PP4/SMK-1 depletion, which is expected, since this complex is indeed required for kinetochore-microtubule attachment and chromosome bi-orientation.

The authors here present the interesting finding that PP4/SMK-1 is required for timely outer kinetochore assembly. They go on to describe how perturbing the timing of outer kinetochore formation leads to delayed chromosome bi-orientation, which is entirely expected. However, they do not address the key question, which is how PP4 may contribute to building the outer kinetochore. In my opinion, while understanding the role of PP4 phosphatases in mitosis is an important endeavor, I don't think this paper provides enough of an advance in that realm to be of broad interest to the field.

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

This manuscript describes a detailed follow-up on a hit from a previously published genetic screen in C. Elegans that looked for genetic interactors of the dynein regulator Nud-2. They determine that the Smk-1 PP4 regulatory and PPH 4.1/4.2 catalytic subunits function in the same pathway and play a role in promoting kinetochore assembly and consequently chromosome alignment and accurate division.

Since relatively little is known about PP4, the data shown here provide a good conceptual advance in the field that is appropriate for JCB. Overall, the data is well presented, of high quality (appropriate for JCB) and easy to follow. The text is likewise generally clear and well written. I am supportive of publication if the authors resolve a few issues.

Major issues:
The authors make extensive use of knockdown and knockout models. However, evidence of silencing or knockout is lacking for PPH 4.1/4.2, KLP-19, wapl, knl1, hcp-4, scc-1 and combination knockdowns. It is a technical detail, yet it seems important to provide direct evidence for the efficiency of gene silencing and/or knockout. Providing this evidence will bolster conclusions related to loss of function and eliminate lingering questions about potential hypomorph phenotypes.

The phenotypes caused by PP4 silencing suggest to the authors that monopolar attachments occur in these cells, resulting in polar chromosome movement followed by eventual bi-orientation. This attachment hypothesis is a major part of the structure of the paper, as it is mentioned twice in the abstract and 2 different sections of the results (Figs 2 and 5). It would strengthen the manuscript if these claims of attachment phenotype were supported by direct imaging rather than inferring their existence based on chromosome movement. If this is not available, then they should soften the conclusions about attachment state and claims of merotely (line 313) and mono-to-bi-orientation conversion (line 329). Also, please explain why since PP4 loss of function would in theory affect both sister kinetochores equally, monopolar attachments result rather than weak bi-polar attachments?

Minor issues:
General comment: The word "inhibition" generally implies blocking function with a chemical, antibody or other means, while leaving protein level intact. We suggest using the word 'knockdown' or 'silencing' when RNAi or genetic knockout is used.

It is unclear why sometimes the histone H2B nomenclature and sometimes HIS-58 nomenclature is used in the figures and legends. If there is a distinction between them, it was unclear to us.

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Line 132-138: We find the text slightly confusing here. Please revise the text and clarify the logic used to justify the co-knockdown of SMK1 and KLP-19 and the conclusions obtained from this experiment.

Line 147: Please describe the experiments/criteria (however briefly) used to test centrosome maturation.

Line 228: Has delayed centromere resolution been previously shown to increase merotelic attachments? Please provide a reference.

Line 270: The authors might consider mentioning the absence of SMK-1 kinetochore localization from figure 2I. If there had been strong kinetochore localization of SMK-1 to begin with, the rescue experiment with the binding mutants would have been more likely to work.

Figure S2D: Please provide the quantification for this figure and provide an inset for the controls.

Figures 2D-E, 4, S1A-D: Please avoid using the combination of green and red to assist colour blind individuals (particularly fig 4).

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Figure 5A: please provide the kymographs for the other conditions shown to facilitate comparison between them.

Figure 5C: Please show control embryos and quantify the percentage of embryos with lagging chromosomes

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HCP-4(CENP-C) and EVH1 mutations on SMK1 perturb HCP-4(CENP-C)-SMK1 interaction in vitro. However, this does not seem to perturb PP4 targeting in cells. I wonder how well HCP-C(CENP-C) is depleted in cells upon RNAi treatment. Is it possible that residual endogenous HCP-4 dimerises with the mutant and rescues the function?

2. In the section where the Ndc80 4A mutant experiment is described (page 10, lines 292-293), this mutant is not introduced with sufficient information. It would help the reader if details of Ndc80 4A were elaborated here with some background information.

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We thank the reviewer for this feedback, and we agree that understanding how PP4 is promoting outer kinetochore assembly is indeed the major question raised by our analysis. This is precisely why we expended significant effort on CENP-C, building a rigorously validated replacement system followed by extensive mutagenesis guided by biochemical reconstitutions. In the revision, we have now added analysis of a structure-guided point mutant engineered in the substrate-binding EVH1 domain of SMK-1 (new Fig. 4F - H). While all our data argue against CENP-C being the relevant target of PP4, the new analysis of the EVH1 domain mutant indicates that substrate recognition by this conserved domain is important for proper chromosome alignment and segregation. We plan to build on this finding to identify the key target(s) of PP4 in future work.

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Regarding RNAi efficiency in our experimental system, we would like to highlight that the *C. elegans* hermaphrodite gonad is uniquely suited for RNAi-mediated depletion because pre-existing protein is continuously packaged into the oocytes that become embryos and is therefore actively removed from the gonad while no new protein is being synthesized (Oegema and Hyman, WormBook 2006: https://doi.org/10.1895/wormbook.1.72.1). Consequently, any protein target is reliably depleted after 48 h, regardless of the intrinsic stability of the protein. Furthermore, RNAi efficiency is ensured by using long dsRNAs (~1 kb), which target many different sites per mRNA. Finally, we deliver *in vitro* synthesized dsRNAs directly by injection, which is the method that produces the most penetrant RNAi.

Regarding specific depletions:

- The dsRNAs for HCP-4, KLP-19, KNL-1, NDC-80, ROD-1, and SKA-3 have been validated in prior studies (Cheerambathur *et al.*, 2017; Cheeseman *et al.*, 2004; Gassmann *et al.*, 2008; Oegema *et al.*, 2001; Powers *et al.*, 2004). For these dsRNAs, as well as for the dsRNAs targeting WAPL-1 and SCC-1, we observe the expected phenotypes, so there can be no doubt that the RNAi is effective. Importantly, with the exception of the PP4 catalytic subunit paralogs for which we use genetic null alleles, none of our conclusions depend on achieving 100% depletion penetrance. The null allele of *pph-4.1* was validated in a previous study (Sato-Carlton *et al.*, 2014), and to generate the null allele of *pph-4.2* we removed the entire open reading frame (see Fig. S1A) to preclude any possibility of residual protein expression. We then performed all possible combinations of knock-out/RNAi to unambiguously demonstrate functional redundancy of the two paralogs.

- The dsRNA we use for replacing endogenous HCP-4 with RNAi-resistant HCP-4 (Figure 4) is shorter than 1 kb and could therefore in principle be somewhat less efficient than the longer dsRNA we use for HCP-4 depletion in Figure 3/Figure S2. In this case we present two experiments that demonstrate efficiency of depletion:

1) RNAi in embryos that do not express RNAi-resistant HCP-4 results in the expected "kinetochore-null" phenotype (Desai *et al.*, 2003; Oegema *et al.*, 2001), i.e. a complete failure to segregate chromosomes (see time point 270 s in Figure 4D).

2) In a new experiment (Figure S3B), we quantified chromosomal levels of endogenously tagged GFP::HCP-4, which shows that GFP::HCP-4 becomes essentially undetectable after RNAi, consistent with the observed kinetochore-null phenotype.

The phenotypes caused by PP4 silencing suggest to the authors that monopolar attachments occur in these cells, resulting in polar chromosome movement followed by eventual bi-orientation. This attachment hypothesis is a major part of the structure of the paper, as it is mentioned twice in the abstract and 2 different sections of the results (Figs 2 and 5). It would strengthen the

manuscript if these claims of attachment phenotype were supported by direct imaging rather than inferring their existence based on chromosome movement. If this is not available, then they should soften the conclusions about attachment state and claims of merotely (line 313) and mono-to-bi-orientation conversion (line 329). Also, please explain why since PP4 loss of function would in theory affect both sister kinetochores equally, monopolar attachments result rather than weak bi-polar attachments?

The high microtubule density in the spindle and the holocentric chromosome architecture, in which microtubule attachments occur along the entire length of sister chromatids, make it impossible to discern by light microscopy whether a chromosome is exclusively attached to only one spindle pole. A chromosome that exhibits pronounced poleward movement must be primarily attached to that pole, but we agree that it cannot be excluded that there could be some microtubules that also connect the chromosome to the other pole. We changed the text in lines 313 (new 321) and line 329 (new 338) accordingly.

When outer kinetochores are ready to engage microtubules in PP4-inhibited embryos after the initial delay, some chromosomes will be positioned/oriented such that they simultaneously attach to both spindle poles, but many chromosomes will first attach to microtubules from one of the spindle poles, as is commonly observed in vertebrate cells. In a control situation, initial mono-oriented attachment occurs early in prometaphase when chromosomes are close to spindle poles, where polar ejection forces are strongest. This limits the extent to which mono-oriented chromosomes move poleward. In PP4-inhibited embryos, chromosomes have already been pushed toward the spindle equator by the time kinetochore-microtubule attachments are formed. Since polar ejection forces are predicted to be weaker at the spindle equator, poleward movement resulting from initial kinetochore-microtubule interactions is more pronounced in PP4-inhibited embryos. We posit that the simultaneous action of polar ejection forces and kinetochore-microtubule attachments in early prometaphase promotes rapid bi-orientation. In PP4-inhibited embryos the two types of chromosome-microtubule interactions become temporally uncoupled from each other, which produces the unusual chromosome scattering phenotype and delays bi-orientation.

Minor issues:
General comment: The word "inhibition" generally implies blocking function with a chemical, antibody or other means, while leaving protein level intact. We suggest using the word 'knockdown' or 'silencing' when RNAi or genetic knockout is used.

To summarize results/conclusions we use "PP4 inhibition" as a general term for perturbing PP4 function regardless of method (RNAi-mediated depletion or genetic null alleles). Whenever we refer to specific experiments in the Results section, we state the exact nature of the inhibition, e.g. *smk-1(RNAi)* or *Δsmk-1.*

It is unclear why sometimes the histone H2B nomenclature and sometimes HIS-58 nomenclature is used in the figures and legends. If there is a distinction between them, it was unclear to us.

For consistency, we replaced "histone H2B" throughout the figures with the *C. elegans* protein names "HIS-58" or "HIS-11", so that it is clear from looking at the figure panels which specific histone H2B paralog is expressed. For readers unfamiliar with *C. elegans* nomenclature, we explain in the figure legends that HIS-58 and HIS-11 correspond to histone H2B.

Figure 1E: No control panel is provided for this set of images. Please show control cells.

Because of space restrictions in the figure and because the null alleles of *pph-4.1* and *pph-4.2* are essentially indistinguishable from the control, we show the corresponding control panel in Figure S1F. Fig. 1E/F and S1F/G show the different RNAi/knock-out combinations. They belong together and are discussed together in the text.

Line 98: We think it more likely that the anaphase bridges arise from the problems with centromere resolution presented later in the manuscript rather than chromosome-spindle attachment. To our knowledge, bridges arise from problems in DNA replication/repair or concatenation. Please provide a reference to support or revise the text.

In *C. elegans*, where microtubule attachments are formed along the entire length of holocentric chromosomes, the consequence of merotelic mis-attachment is that chromosomes become stretched out along the spindle axis in anaphase. This is not the case for merotelic attachments on monocentric chromosomes, where a chromatid with a merotelically attached kinetochore lags behind the rest of the separating chromatids during anaphase. This section of the text now states that anaphase chromatin bridges are a hallmark of attachment errors in holocentric organisms, and we added two appropriate references (Stear and Roth, 2002; Gassmann *et al.*, 2008).

Line 132-138: We find the text slightly confusing here. Please revise the text and clarify the logic used to justify the co-knockdown of SMK1 and KLP-19 and the conclusions obtained from this experiment.

We show these results here because the phenotype we observe in the SMK-1/KLP-19 double knockdown (essentially all chromosomes temporarily move to one or the other spindle pole) supports the conclusion that PP4 inhibition induces a prolonged mono-orientated state, which is further exacerbated when polar ejection forces are removed.

Line 147: Please describe the experiments/criteria (however briefly) used to test centrosome maturation.

Defects in centrosome maturation would result in spindles with reduced β- and γ-tubulin signal at centrosomes (i.e. centrosomes would look small when looking at these markers, which we use throughout the paper). We observe none of these defects even in the most stringent PP4 inhibition conditions. We also directly assessed the capacity of centrosomes to nucleate microtubules and found it to be normal in PP4-inhibited embryos. We have changed the text to explicitly refer to these criteria.

Line 228: Has delayed centromere resolution been previously shown to increase merotelic attachments? Please provide a reference.

A mitotic phenotype in which centromere resolution is delayed (rather than permanently impaired) has not been described previously. However, since perturbations that impair the back-to-back geometry of sister kinetochores make holocentric chromosomes susceptible to merotely (e. g. Stear and Roth, 2002), we tentatively state that delayed centromere resolution in PP4-inhibited embryos is "likely" to elevate merotely.

Line 270: The authors might consider mentioning the absence of SMK-1 kinetochore localization from figure 2I. If there had been strong kinetochore localization of SMK-1 to begin with, the rescue experiment with the binding mutants would have been more likely to work.

We performed an additional experiment that further supports the view that CENP-C is not the relevant target of PP4 (see response to reviewer 1) and have updated the paragraph that included line 270.

Figure S2D: Please provide the quantification for this figure and provide an inset for the controls.

We now show the quantification of chromosomal DHC-1::GFP levels in Figure S2D.

The inset of *smk-1(RNAi)* shows that DHC-1::GFP accumulates on both sister kinetochores in late prometaphase, which is to illustrate that outer kinetochores have fully assembled (dynein is the outermost kinetochore component). Control chromosomes at this time point are fully aligned on the metaphase plate, and it is therefore not possible to show a control inset with an isolated chromosome.

Figures 2D-E, 4, S1A-D: Please avoid using the combination of green and red to assist colour blind individuals (particularly fig 4).

We simulated protanopia- and deuteranopia colorblindness for all figures and changed colors/made labeling more explicit where there was ambiguity, particularly in Figure 4.

Figure 3C: Please show the channels individually and also overlaid. It is hard to see both together, particularly for the mutant.

The GFP::HCP-3 channels are shown in separate in Figure 3A (first and second images from top left; images in Figure 3C are magnified versions). The images in Figure 3C are shown simply to accompany the schematic on the right, in which we explain how we performed the line scans to quantify resolution.

Figure 3G: Please show control embryos for this experiment

A corresponding control embryo for this condition is shown in Figure 2A and in supplemental Video 1. We opted to not show it again here because the emphasis in this figure is on the difference between the single *scc-1(RNAi)* depletion and the *scc-1(RNAi);smk-1(RNAi)* double depletion. Note, however, that the control embryo data is shown in the graph on the right (Figure 3H).

Figure 5A: please provide the kymographs for the other conditions shown to facilitate comparison between them.

Kymographs for the control and *smk-1(RNAi)* condition are shown in Figure 1C and Figure S1B.

Figure 5C: Please show control embryos and quantify the percentage of embryos with lagging chromosomes

The purpose of this panel is to show that chromosomes that move to spindle poles in the Ska/SMK-1 double inhibition, which is described and quantified in Figure 5A and B, consist of cohesed sister chromatids, i.e. sister chromatids co-segregate to spindle poles prior to anaphase onset. A control embryo expressing the GFP::HCP-3 marker is shown in Figure 3A and supplemental Video 7.

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

The kinetochore is a large multi-subunit assembly that physically couples chromosomes to the spindle microtubules and harnesses the spindle-associated forces to achieve chromosome segregation during cell division. While the 'inner' kinetochore complexes provide the specificity for kinetochore assembly, the 'outer' kinetochore complexes directly interact with microtubules to facilitate chromosome-spindle coupling. Timely kinetochore assembly is critical to ensure accurate chromosome segregation. Here, in this manuscript, Rocha et al., identify PP4, a protein phosphatase known for its post-mitotic functions in longevity and stress resistance, as an important regulator that promotes outer kinetochore assembly prior to nuclear envelope breakdown. This is an important finding in the field, particularly considering how little we know about the temporal regulation of functional kinetochore assembly and the PP4-mediated regulation of cell division. Overall the conclusions of this work are well supported by high-quality work, and the manuscript is clearly written. I only have a couple of minor queries/suggestions.

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To address this point, we show three experiments, the first two of which are already described above in response to reviewer 2's concern about depletion efficiencies:

1) RNAi in embryos that do not express RNAi-resistant HCP-4 results in the expected "kinetochore null" phenotype (Desai *et al.*, 2003; Oegema *et al.*, 2001), i.e. a complete failure to segregate chromosomes (see time point 270 s in Figure 4D).

2) In a new experiment (Figure S3B), we quantified the chromosomal levels of endogenously tagged GFP::HCP-4, which shows that GFP::HCP-4 becomes essentially undetectable after RNAi, consistent with the observed kinetochore null phenotype.

3) In another new experiment (Figure S3D), we observe severe chromosome segregation defects when using an RNAi-resistant HCP-4 mutant that cannot be imported into the nucleus, presumably because one of the FxxP motif mutations (F438A) disrupts a predicted NLS. This mutant corresponds to mutant number 6 in Figure 4B, which was previously mentioned in the text. Importantly, despite the absence of nuclear localization, mutant number 6 localizes normally to prometaphase chromosomes, which shows that it is expressed at similar levels to transgenic wild-type HCP-4. The fact that we can successfully replace endogenous HCP-4 with a transgenic mutant that produces a phenotype serves as a control that validates the molecular replacement approach.

Taken together, these experiments argue against the possibility that the failure to observe a phenotype with HCP-4 mutant number 9 (Figure 4D, E; Figure S3E) is due to insufficient depletion of endogenous HCP-4.

2. In the section where the Ndc80 4A mutant experiment is described (page 10, lines 292-293), this mutant is not introduced with sufficient information. It would help the reader if details of Ndc80 4A were elaborated here with some background information.

The character limit of the JCB Report format make it difficult to introduce this mutant in more detail, and we will therefore have to refer the reader to the original study (Cheerambathur *et al.*, 2017).

December 26, 2022

RE: JCB Manuscript #202208154R

Dr. Reto Gassmann
Institute for Molecular and Cell Biology
Rua Alfredo Allen, 208
Porto, Porto 4200-135
Portugal

Dear Dr. Gassmann,

Thank you for submitting your revised manuscript entitled "Nuclear-enriched protein phosphatase 4 ensures outer kinetochore assembly prior to nuclear dissolution." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

2) Figure formatting: Reports may have up to 5 main text figures. Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please 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.

3) 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, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

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

4) 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."

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

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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.).

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

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

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11) 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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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 electron microscopy and mass spectrometry data in appropriate public databases.

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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:

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

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