Interphase microtubule disassembly is a signaling cue that drives cell rounding at mitotic entry

Kévin Leguay, Barbara Decelle, Islam Elkholi, Michel Bouvier, Jean-Francois Côté, and Sébastien Carréno

Corresponding Author(s): Sébastien Carréno, University of Montreal

Transaction Report:

Revision 0

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

In this manuscript, Leguay et al. identify the upstream signal that regulates SLK-dependent phosphorylation and consequent activation of ERM proteins in mitosis, which has previously been shown to regulate mitotic cell rounding. By screening a library of small molecules in HEK293T cells, they uncover that microtubule disassembly induces ERM activation. They subsequently demonstrate that disassembly of interphase microtubules during mitotic entry acts as a trigger for ERM activation and is needed for mitotic cell rounding. Based in part on previous findings, they show this involves a signaling cascade starting with the RhoGEF GEF-H1 (known to be inhibited by its binding to microtubules) and downstream Rho-dependent activation of SLK. Finally, the authors show that GEF-H1 acts in parallel with another RhoGEF, Ect2, in the mitotic activation of ERM proteins and mitotic cell rounding.

Major comments:

Overall, the work presented in this manuscript is thorough and clearly presented, and the evidence brought forward convincingly fits the proposed model. I do have some concerns that need to be addressed to fully support the conclusions from the authors.

1. The authors quantify the ratio between cell height and width (which they define as sphericity) as a measure for cell rounding, but this approach has several concerns. It is not clear how cell width is determined and the performed measurements will be greatly determined by which cell axis is used to measure this (and this appears to be the length across the arbitrary x-axis). To better understand the shown phenotype, alternative measures for cell rounding that are more conventionally used should be included, at least showing cell height also separately from the

cellular shape within the xy plane (the latter can be either cell area/length of short or long axis/perimeter/circularity). Furthermore, in order to truly be able to interpret the data on defects in mitotic cell rounding, the authors should compare mitotic cell shape with the shape of the same cell in interphase prior to entering mitosis (which can be easily done in live-cell experiments, with for instance membrane markers).

2. The model proposed in this manuscript to a great extent depends on pre-existing knowledge from literature (e.g. GEF-H1 and Rho activation following microtubule disassembly, ref 20 and 21; nocodazole-induced cell rounding through GEF-H1, ref 20; Rho-mediated Slk activation, ref 18; Slk/ERM-dependent mitotic cell rounding, ref 2,3). While this is on itself is not problematic, several key components of their model should also be experimentally tested in their cellular system. Most importantly, the authors propose that SLK depletion disturbs mitotic rounding through the absence of ERM activation; but although the role of ERM proteins in mitotic rounding has been demonstrated in Drosophila cells, mammalian cells depleted of ERM proteins were previously shown not to have any defect in mitotic cell rounding (Machicoane et al., 2014). The authors should therefore perform experiments with ERM depletion and phospho-dead/mimetic mutants to test whether SLK truly functions through ERM in mitotic rounding in HEK293T cells. Similarly, the authors should show that disruption of microtubules indeed leads to activation of Rho in these cells.

3. In Fig. 3, the authors use low levels of Taxol to prevent interphase microtubules disassembly and to test whether this prevents ERM activation and mitotic cell rounding. These experiments lack controls that interphase microtubule disassembly is truly inhibited, which should be included. Furthermore, as stated by the authors "interphase microtubule disassembly frees tubulin dimers that can then re-polymerize to form the mitotic spindle", and it is therefore unclear how this taxol treatment would still allow formation of the mitotic spindle (Fig 3a).

Minor comments:

4. For some WBs, quantifications are lacking. This is especially important to include because the total ERM levels also seem to be affected by microtubule targeting drugs (e.g. 1E)

5. Knockdown efficiencies for siRNA SLK #1 and SLK #2 (3MN, 4HI) are not shown (only for shRNA)

6. The representation of data can sometimes be improved: several representative IF images are relatively small, making it difficult to see the entire contour of the cell (e.g. 4F); individual channels are sometimes not properly visible in merged images, which for instance makes it is difficult to see the effect of nocodozole on tubulin in 2J; and some of the shown examples do not clearly represent the quantified data.

7. Introduction and discussion are lacking

3. Significance:

Significance (Required)

This study couples existing knowledge on the role of ERM proteins in mitotic rounding, Rho/SLK-dependent ERM phosphorylation, and activation of the RhoGEF GEF-H1 following microtubule disassembly, to demonstrate how activation of ERM proteins and consequently mitotic rounding are established by the disassembly of interphase microtubules as cells enter

mitosis. It thereby shows that interphase microtubule disassembly is a cue that drives ERM-mediated rounding of mitotic cells. While all individual components of this pathway were already established, this study connects them and demonstrates their importance in mitotic rounding. These findings will be relevant to an audience interested in understanding the molecular mechanisms underlying morphological changes of mitotic cells.

Field of expertise: cell adhesion, mitotic cell rounding

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

ERM family proteins have been shown to be activated in early mitosis to drive mitotic rounding, but the molecular events that trigger ERM activation are not clear. Here, Leguay and colleagues show that microtubule disassembly can induce ERM phosphorylation through the microtubule-binding GEF-H1. They then show that microtubule disassembly and GEF-H1 are required for mitotic ERM phosphorylation and cell rounding in HEK293T cells and that this pathway works in synergy with Ect2 to control mitotic rounding through the activation of RhoA.

Major comments

This is an elegant and well-presented study with convincing results that justify the conclusions. There are a couple of important issues that need to be addressed before publication:

• The most worrying point is that the authors appear to switch between using siRNA and shRNA knockdown for SLK and GEF-H1. The authors don't draw attention to this in the text or mention why different methods are used. For example, for SLK: shRNA is used in figure 2A, siRNA in figure 2J-K, shRNA in fig 3I-J and siRNA in figure 3M-N and then siRNA in figure 4H. For figure 3, the converse experiments are presented in the supplement but not for the others.

Worryingly, where both sets are included, the results are slightly different: in Fig 3J, the increase in pERM from interphase to mitosis is not significant with shRNA, while in Fig S3D, using siRNA, it is. Although this difference doesn't change the overall conclusions, it makes it look as though the authors have tried both methods and picked the one with the most convincing result for the main paper. In addition, while the shRNA knockdown is validated by the blot in figure 2A, the siRNA knockdown is not. If the authors want to use two different methods to deplete SLK, both need to be validated by western blot and where possible, the data from both methods shown in the supplement. The authors also need to make it clear in the text where they are switching methods and why.

• Much of the conclusions in figures 3 and 4 come from measurements of pERM levels at the plasma membrane in mitosis, however there is not enough information given about how these measurements are made. How was the plasma membrane identified (especially in interphase conditions where pERM levels are very low)? Was intensity measured all around the membrane or just at certain points? Was it the averaged per cell? It's stated that 'p-ERM signal intensity at the plasma membrane is normalized to interphase cells treated with vehicle'. Does this mean that they are normalising across different conditions? It's hard to ensure identical imaging conditions for different samples, so was any kind of internal normalisation also applied? Eg. to background or cytoplasmic levels?

• This work identifies microtubule disassembly and GEF-H1 as triggers for mitotic rounding but proposes that they ultimately work by activating RhoA (alongside Ect2). In the model and text the authors apply that the main function of RhoA is to activate SLK and ERMs rather than ROCK/myosin. This is based on figure 4H-I, where they show that SLK depletion has a greater effect on mitotic cell shape than ROCK inhibition and that there is no synergy between them. The relatively mild effect of ROCK inhibition is at odds with previous studies in different cell types (eg Maddox 2003, Matthews 2012) that show Y27 to block mitotic rounding to a similar extent to RhoA inhibition. To firm up this conclusion, the authors should show that RhoA activation (eg using CN03) directly increases pERM levels in IF or western blot. It would also be great to compare with p-Myosin II in this context.

Minor comments

• Figure 1G is not quantified. Also, the images are very small and the zoom is confusing - it's not clear what we are looking at without the merge.

• Figure 2G: the authors state that GEF-H1 depletion blocks Nz-induced ERM phosphorylation. From their blots there is still an increase, it is just smaller. They should change the text to state that it 'decreases' the effect.

3. Significance:

Significance (Required)

This work identifies a novel mechanism for mitotic cell rounding: via the disassembly of interphase microtubules and GEF-H1. This adds an important missing piece to the puzzle of how

ERM proteins are activated in mitosis, building on the previous works from this team and others. This work is likely to be of great interest to cell biologists interested in mitotic rounding, as well as those more generally interested in cell division, the acto-myosin cytoskeleton and Rho GTPases.

Referee Cross-commenting

I agree with the comments of R1 and R3.

• R1 requests better quantification of cell shape and comparison between interphase and mitosis within the same cell - I completely agree.

• Both R1 & R3 request further controls to demonstrate the effects of taxol on interphase microtubules and the mitotic spindle - I agree this is very important

• R1 also asks for experimental tests of several key aspects of the model: the links between MTs and RhoA and SLK and ERM. Interestingly it was the link between RhoA and SLK/ERM that I picked up on but my point was similar. It's important to test these elements of the model in this system.

• Reviewer 3's suggestion to investigate substrate adhesion and KANK is very interesting and I agree this should be mentioned. However gathering data on this likely to require substantial work and I would say is beyond the scope of this current work.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This report used a small molecule screen to identify microtubule (MT) inhibitors as activators of ERM proteins, and consolidated the screen results with several specific follow-up analyses of different inhibitors, cell types, treatment times, and treatment concentrations. Applied to cultured

interphase mammalian cells, MT inhibition increased ERM protein phosphorylation and cell rounding. The effect on ERM protein phosphorylation was abolished by SLK depletion or Rho-GTP inhibition, which both also reduced a lower level of phosphorylation detected without MT inhibitor addition. Depletion of GEF-H1, a Rho-GEF known to be inhibited by MTs, specifically and substantially reduced the phosphorylation that occurred in response to the MT inhibition. Since interphase MTs are known to be disassembled upon entry into mitosis, the authors stabilized MTs with taxol and found that metaphase cells had reduced rounding and reduced ERM protein phosphorylation, effects mimicked by depletion of GEF-H1, Rho-GTP, or SLK, and suppressed by a chemical activator of Rho. Finally, GEF-H1 is shown to work in parallel Ect2, the cell cycle activated Rho-GEF, for ERM protein phosphorylation and cell rounding. The identification of cell cycle regulation of GEF-H1 through MT disassembly and the downstream effects of this signaling on ERM phosphorylation and cell rounding are the main advances of the paper, and should be of substantial interest to cell biologists. Overall, the study's conclusions are supported by a variety of approaches, tests of alternate mechanisms, appropriate controls, and thorough quantifications and statistical analyses. It could be improved by addressing the points below.

1. Can an effect of taxol on MTs be detected? The spindles shown look similar with or without taxol. Are there delays in the disassembly of interphase MT arrays that could explain the effect of taxol? Additional data could help clarify whether the effect of taxol is consistent with the authors' interpretations.
2. Mitotic rounding can involve focal adhesion disassembly, and a reduction of attachment to the substrate, presumably reflected by the authors' assays of cell X-Z dimension ratios in side views (the sphericity assays). The Bershadsky lab recently showed that GEF-H1 seems to be specifically regulated by KANK-based MT attachment to focal adhesions (PMID: 31114072). For example, KANK depletion resulted in GEF-H1 activation without loss of the full MT network. The current work could be further fleshed out by investigating whether depletion of KANK increases sphericity and ERM phosphorylation in interphase cells. Is it the general remodeling of the MT cytoskeleton or the specific loss of MT-FA attachments that are important? Experimental investigation of this point may or may not go beyond the scope of the current paper, but the KANK-based mechanism of GEF-H1 regulation should be discussed at minimum. Also, the cell culture substrate should be added to the Methods section.

3. Significance:

Significance (Required)

Mitotic cell rounding is an important step of the cell cycle. It involves cell cycle signals that activate actomyosin contractility plus actin attachment to the plasma membrane via ERM proteins. ERM proteins are known to be activated by SLK phosphorylation downstream of Rho-GTP, but a linkage of this pathway to the cell cycle was unknown. This report identifies two molecular mechanisms linking ERM activation to the cell cycle for cell rounding. It should be of substantial interest to cell biologists.

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Corresponding author(s): Sébastien, Carréno

1. General Statements

In our study, we resolved a long-standing question on how the cell cycle controls rounding of cells at mitotic entry. We discovered that disassembly of the array of interphase microtubule directs cortical actin reorganization to prompt cell rounding.

We thank the editors of Review Commons that handled our manuscript and the reviewers for their suggestions that will undeniably improve its quality. We are happy that the three reviewers found that our work:
-is thorough and clearly presented, and the evidence brought forward convincingly fits the proposed model. (Reviewer 1)
-is an elegant and well-presented study with convincing results that justify the conclusions. (Reviewer 2)
-conclusions are supported by a variety of approaches, tests of alternate mechanisms, appropriate controls, and thorough quantifications and statistical analyses. (Reviewer 3)

In addition, we are pleased that the reviewers acknowledged that:
-These findings will be relevant to an audience interested in understanding the molecular mechanisms underlying morphological changes of mitotic cells. (Reviewer 1)
-This work is likely to be of great interest to cell biologists interested in mitotic rounding, as well as those more generally interested in cell division, the acto-myosin cytoskeleton and Rho GTPases. (Reviewer 2)
-It should be of substantial interest to cell biologists. (Reviewer 3)

2. Description of the planned revisions

Insert here a point-by-point reply that explains what revisions, additional experimentations and analyses are <u>planned</u> to address the points raised by the referees.

<u>Reviewer 1-1a</u>: The authors quantify the ratio between cell height and width (which they define as sphericity) as a measure for cell rounding, but this approach has several concerns. It is not clear how cell width is determined and the performed measurements will be greatly determined by which cell axis is used to measure this (and this appears to be the length across the arbitrary x-axis).

We agree with reviewer 1 that measurement of cell sphericity can be affected by the x-axis chosen. We apologize for our lack of precisions because we did take this concern into consideration when we determined cell width to calculate sphericity. Indeed, cell width was calculated as the average of the longer axis of the cell and its orthogonal in order to not be biased

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by the orientation of cells in the x/y plane. This will be better explained in the material and methods section of the revised manuscript.

Reviewer 1-1b: To better understand the shown phenotype, alternative measures for cell rounding that are more conventionally used should be included, at least showing cell height also separately from the cellular shape within the xy plane (the latter can be either cell area/length of short or long axis/perimeter/circularity). Furthermore, in order to truly be able to interpret the data on defects in mitotic cell rounding, the authors should compare mitotic cell shape with the shape of the same cell in interphase prior to entering mitosis (which can be easily done in live-cell experiments, with for instance membrane markers).

We thank Reviewer 1 for these suggestions that will provide a better description of the shown phenotypes. We will include the following suggested measurements in each figure of the original manuscript that were reporting sphericity. As an example, we attach to this plan new figures regarding taxol treatment, corresponding to Fig 3B of the original manuscript. As suggested by the Reviewer 1 these figures include now:

-cell height (Fig R1B)
-the average length of the longer axis (L) and its orthogonal (O) (Fig R1C).

We also agree with reviewer 1 and reviewer 2 (in the referee cross-commenting section), that a better description of interphase cells would help assessing how cells round-up in metaphase. To address this, we will include side to side measurements (sphericity, cell height and axis average length) of interphase (I) and metaphase (M) cells (Fig R1A-C). We will perform these measurements in fixed samples since this method allows measurement of a larger number of cells than with time-lapse experiments. Therefore, the presented data will be more quantitative.
In addition, and as represented in Fig R1D, we will quantify cell shape transformation from interphase to metaphase by measuring the mean height ratio of metaphase to interphase cell populations in at least three independent experiments. This representation better quantifies cell rounding from the interphase cell population to the metaphase cell population.
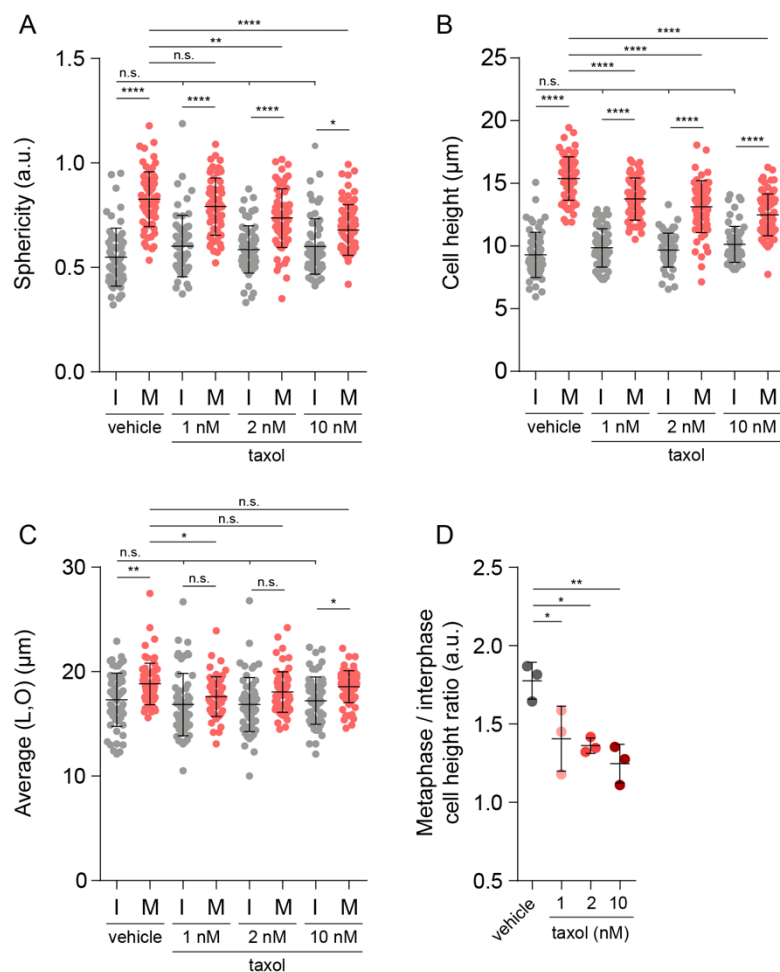
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Figure R1: Cell sphericity and cell height upon taxol treatment.

(A-C) Cell sphericity (A), cell height (B) and cellular shape within the x/y plane (C) in interphase (I) and metaphase (M) cells. Cells were treated with vehicle or taxol for 90 min before fixation. Cell sphericity (A) was determined as a ratio of cell height to cell width (determined as the average between the longer axis (L) and its orthogonal (O)). Data for mitotic cells are already presented in Fig 3B of our manuscript, but we propose to add measurements for interphase cells in the revised version.

(D) Cell height ratios of metaphase to interphase cell populations after taxol treatment.

Quantifications represent the mean +/- s.d. of three independent experiments. Dots represent individual cells (A-C) or independent experiments (D). P values were calculated using Sidak's multiple comparisons test with a single pooled variance (A-C) or a two-tailed t test (D). *, P < 0.05. **, P < 0.01. ****, P < 0.0001. ns, not significant.

Reviewer 1-2a: The model proposed in this manuscript to a great extent depends on pre-existing knowledge from literature (e.g. GEF-H1 and Rho activation following microtubule disassembly, ref 20 and 21; nocodazole-induced cell rounding through GEF-H1, ref 20; Rho-mediated Slk activation, ref 18; Slk/ERM-dependent mitotic cell rounding, ref 2,3). While this is on itself is not problematic, several key components of their model should also be experimentally tested in their cellular system. Most importantly, the authors propose that SLK depletion disturbs mitotic rounding through the absence of ERM activation; but although the role of ERM proteins in mitotic rounding has been demonstrated in Drosophila cells, mammalian cells depleted of ERM proteins were previously shown not to have any defect in mitotic cell rounding (Machicoane et al., 2014). The authors should therefore perform experiments with ERM depletion and phospho-dead/mimetic mutants to test whether SLK truly functions through ERM in mitotic rounding in HEK293T cells.

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We agree that while the roles of ERM proteins and SLK in rounding of mitotic cells have been clearly established in Drosophila (dMoesin and Slik), these roles are less clear in mammalian cells.

Machicoane et al., 2014 reported that mammalian cells depleted for SLK did not display lack of cell rounding. Accordingly, movies of SLK RNAi cells shown in this article showed that metaphase cells appear to round up. However, this was observed by phase contrast time-lapse microscopy, a technic that can give indication if cells round-up to a certain extent but cannot quantitatively measure the 3D shape of mitotic cells. In our manuscript, we used 3D reconstruction after confocal microscopy to measure sphericity. By doing so, we established that SLK controls metaphase cell sphericity (Fig 3 M,N). In the same article, Machicoane et al., 2014 did not observe an apparent lack of cell rounding when the three ERMs are co-depleted. However, this was not quantified by 3D reconstruction after confocal microscopy. In contrast to Machicoane observations, Toyoda et al, 2017, (PMID: 29097687) showed that Ezrin RNAi silencing led to a decrease of metaphase cortical tension in HeLa cells (Supplementary Data 1, PMID: 29097687). Since increase of cortical tension at mitosis onset provides an essential force that prompts metaphase cell rounding, their data suggest that the role of ERM proteins for metaphase rounding is conserved in mammals.

Nevertheless, we agree that assessing the role of ERMs in metaphase cell rounding using 3D reconstruction after confocal microscopy would strengthen some conclusions of our study. But loss-of-function approaches of ERM proteins are very challenging because of the functional redundancy between ezrin, radixin and moesin. At a first attempt to perform an ERM loss-of-function, we aimed to stably co-deplete the three ERMs by lentiviral shRNA delivery. This method allows to select stable knock-down cells. After selection we found that stable co-depletion of ERMs strongly affected cell survival and growth of HEK293T cells. This made the characterization of mitotic cell rounding impossible. To overcome this difficulty, we are now proposing to test the role of ERMs for mitotic cell rounding, by transiently co-depleting ezrin, radixin and moesin using corresponding siRNA. If this siRNA approach allows a satisfactory co-depletion of the three ERMs and a good viability of the transiently co-depleted cells, we will be able to include the results of this experiment in the revised version of the manuscript.

As an alternative approach to test the role of ERM for mitotic rounding, and as proposed by Reviewer 1, we will perform rescue experiments using an ezrin phospho-mimetic (T567D) mutant. If ezrinT567D rescues metaphase cell rounding upon SLK depletion it will strengthen the conclusions that SLK controls mitotic rounding through ERM activation. If not, it will indicate that in addition to ERMs, SLK activates other still unknown protein(s) that participates to mitotic cell rounding. If it is the case, this will be discussed in the revised version of the manuscript.

Reviewer 1-2b: Similarly, the authors should show that disruption of microtubules indeed leads to activation of Rho in these cells.

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As proposed by Reviewer 1 and supported by reviewer 2 in the referee cross-commenting section, we will test if disruption of microtubules using nocodazole leads to RhoA activation in HEK293T cells by using a rhotekin-RBD pull down assay. We indeed already obtained in a first experiment the evidence that nocodazole treatment activates RhoA in HEK293T cells using this approach (Figure R2).
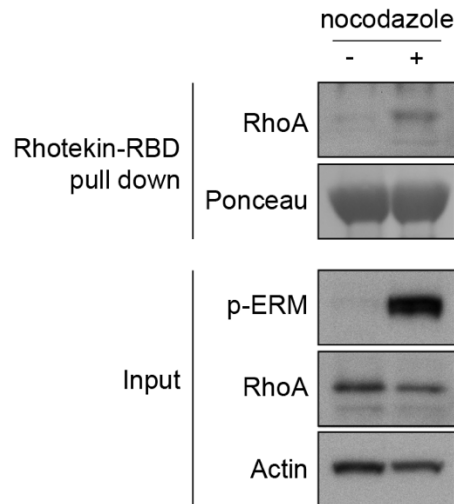
Figure R2: Rhotekin-RBD pull down in HEK293T cells treated with nocodazole.
GST-Rhotekin-RBD pull down assay was performed in HEK293T cell lysates treated with vehicle or 1 µM nocodazole for 15 min and incubated for 1h at 4ºC with GST-Rhotekin-RBD beads. Total lysate (input) is shown at the bottom. GST-Rhotekin-RBD amount is evaluated by Ponceau staining and RhoA amount by western-blotting.

Reviewer 1-3. In Fig. 3, the authors use low levels of Taxol to prevent interphase microtubules disassembly and to test whether this prevents ERM activation and mitotic cell rounding. These experiments lack controls that interphase microtubule disassembly is truly inhibited, which should be included. Furthermore, as stated by the authors "interphase microtubule disassembly frees tubulin dimers that can then re-polymerize to form the mitotic spindle", and it is therefore unclear how this taxol treatment would still allow formation of the mitotic spindle (Fig 3a).

We agree with the reviewers that if the disassembly of interphase microtubules is perturbed by low doses of taxol, it should lower the availability of tubulin dimers and thereby affects to some extent the formation of the mitotic spindle.

We used the same low concentrations of taxol to perturb the disassembly of interphase microtubules at mitosis entry than Mchedlishvili and colleagues (2018). They already have intensively characterized this treatment in HeLa cells in their article. However, we did not mention in our manuscript if low dose of taxol affected mitosis in HEK293T cells. We apologize for this omission, and we will add Figure R3 in the revised version of our manuscript to address this issue. In this figure, we showed that low dose of taxol (10 nM) increased the number of mitotic cells compared to control (Fig R3B) indicating that entrance into mitosis is perturbed. In addition, we measured an increase of monopolar spindles in metaphase (Fig R3C) demonstrating that the

effect of low doses of taxol is consistent with our interpretations, since as one could expect, it affected the formation of the mitotic spindle.
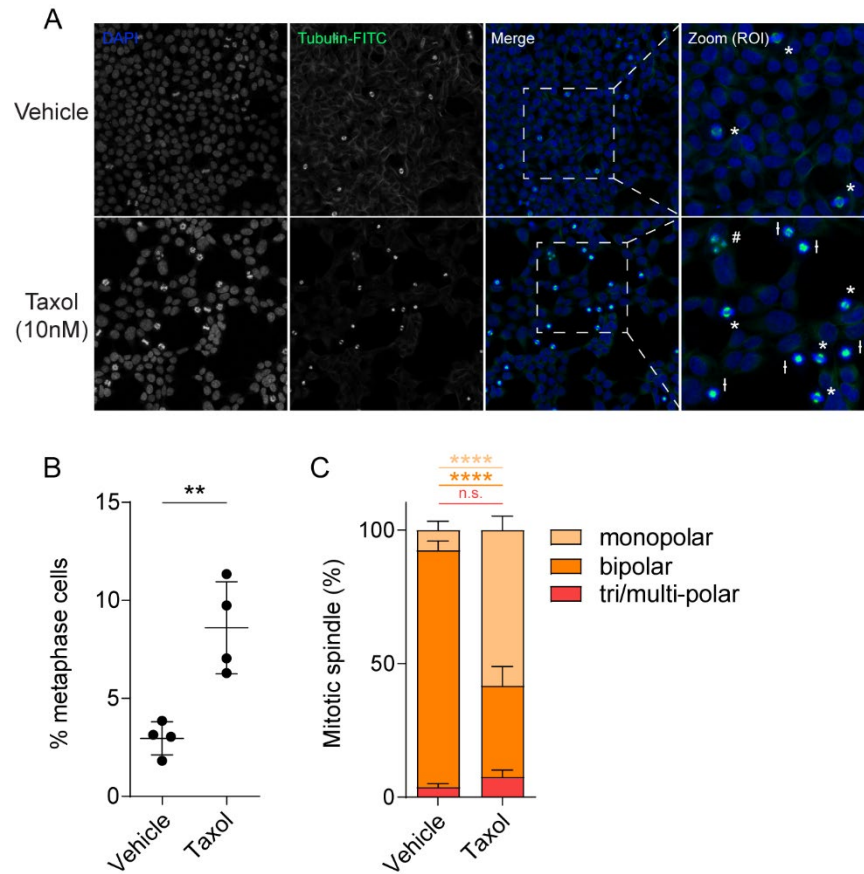
Figure R3: Mitotic index following taxol treatment.
(A) Immunofluorescence of HEK293T cells treated with vehicle or taxol (10 nM). (B) Percentage of HEK293T metaphase cells after vehicle or taxol treatment for 90 min. (C) Characterization of mitotic spindle (monopolar, bipolar, tri/multi-polar). P values were calculated using a two-tailed t test. **, P < 0.01. ****, P < 0.0001. n.s., not significant.

Reviewer 2-1. The most worrying point is that the authors appear to switch between using siRNA and shRNA knockdown for SLK and GEF-H1. The authors don't draw attention to this in the text or mention why different methods are used. For example, for SLK: shRNA is used in figure 2A, siRNA in figure 2J-K, shRNA in fig 3I-J and siRNA in figure 3M-N and then siRNA in figure 4H. For figure 3, the converse experiments are presented in the supplement but not for the others. Worryingly, where both sets are included, the results are slightly different: in Fig 3J, the increase in pERM from interphase to mitosis is not significant with shRNA, while in Fig S3D, using siRNA, it is. Although this difference doesn't change the overall conclusions, it makes it look as though the authors have tried both methods and picked the one with the most convincing result for the main paper. In addition, while the shRNA knockdown is validated by the blot in figure 2A, the

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siRNA knockdown is not. If the authors want to use two different methods to deplete SLK, both need to be validated by western blot and where possible, the data from both methods shown in the supplement. The authors also need to make it clear in the text where they are switching methods and why.

We apologize for this apparent lack of clarity. As noticed by Reviewer 2, we used both siRNA and shRNA to perform our knock-down experiments. We agree that switching between siRNA and shRNA may appear confusing even if it as stated by Reviewer 2, it does not change our overall conclusions. We will modify the revised manuscript to make clear where and why we are using siRNA or shRNA. We will also switch Fig 3I-J and Fig S3C-D so that Figure 3 will show data solely obtained using siRNA. Finally, western blot showing knockdown efficiencies for siRNA against SLK will be added in the revised manuscript.

Reviewer 2-2. Much of the conclusions in figures 3 and 4 come from measurements of pERM levels at the plasma membrane in mitosis, however there is not enough information given about how these measurements are made. How was the plasma membrane identified (especially in interphase conditions where pERM levels are very low)? Was intensity measured all around the membrane or just at certain points? Was it the averaged per cell? It's stated that 'p-ERM signal intensity at the plasma membrane is normalized to interphase cells treated with vehicle'. Does this mean that they are normalising across different conditions? It's hard to ensure identical imaging conditions for different samples, so was any kind of internal normalisation also applied? Eg. to background or cytoplasmic levels?

We agree with Reviewer 2 that some information is missing concerning how p-ERM levels were measured at the plasma membrane. The following information will be added to the revised manuscript: Images acquisitions were performed with identical imaging conditions across the same experiment. p-ERM signals were measured all around the plasma membrane using the segmented line tool from Image J software. p-ERM intensity is represented as the average of p-ERM levels at the plasma membrane per cell, in which the background was subtracted. In experiments were p-ERM levels were quantified in both interphase and metaphase cells, p-ERM levels per cell were normalized to the average of p-ERM levels in interphase control cells (vehicle or si/shRNA non-target). Thus, the average of normalized p-ERM levels in control interphase cells is equal to 1.

In addition, to verify that p-ERM levels can be compared in the different conditions of the same experiment, we routinely assess the background signals from each condition that should be comparable. As an example, Fig R4 represents the background signals measured for Fig 3D of the original manuscript. This shows that background signals are similar across every condition indicating that p-ERM levels can be compared.

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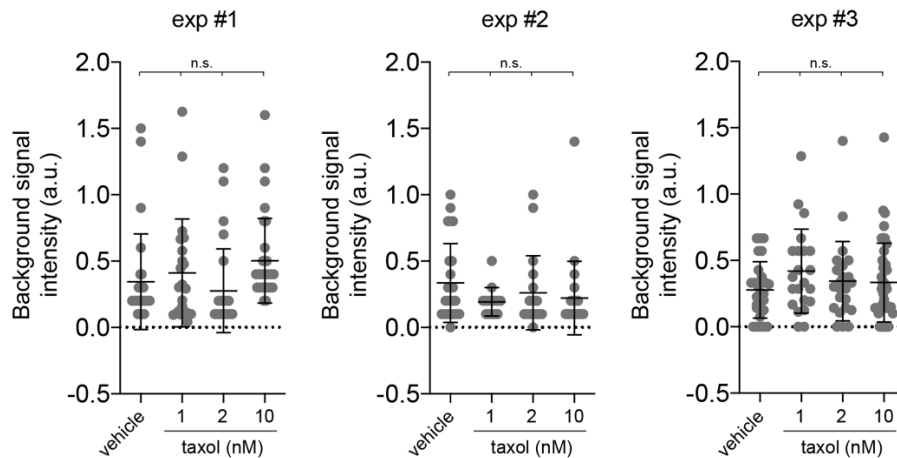
Figure R4: Background signal intensity measured by immunofluorescence.
Background signal intensity from three independent experiments (exp #1-3) in whose HEK293T cells were treated with vehicle or taxol for 90 min before fixation and immunostaining with p-ERM antibody (see Fig 3C-D of the original manuscript). P values were calculated using a two-tailed t test. n.s., not significant.

Reviewer 2-3a. This work identifies microtubule disassembly and GEF-H1 as triggers for mitotic rounding but proposes that they ultimately work by activating RhoA (alongside Ect2). In the model and text the authors apply that the main function of RhoA is to activate SLK and ERMs rather than ROCK/myosin. This is based on figure 4H-I, where they show that SLK depletion has a greater effect on mitotic cell shape than ROCK inhibition and that there is no synergy between them.

We apologize if some of our writing was misleading. We did not want to imply that the main function of RhoA is to activate SLK and ERMs rather than ROCK/myosin. Indeed, we even concluded in the manuscript that *'Ect2 and GEF-H1 both engage RhoA that relays the cell-cycle signals to its two effectors, ROCK and SLK. ROCK takes in charge the generation of cortical acto-myosin forces while SLK activates ERMs that couple these forces to the plasma membrane (Fig 5). This integrated signaling network ensures that the multiple events that promote the reorganization of the actin cortex are properly coordinated and synergized at mitosis onset to drive cell rounding.'*
However, we realize that our model drawn in Figure 5 could be misleading. The dashed arrows from RhoA to ROCK to myosin light chain 2 may imply that ROCK is less important than SLK-ERM for mitotic rounding. That was not our intention, we choose the dashed arrows because this ROCK pathway has been characterized before our manuscript. We will change these dashed arrows to plain arrows in the revised version of the manuscript.

Reviewer 2-3b. The relatively mild effect of ROCK inhibition is at odds with previous studies in different cell types (eg Maddox 2003, Matthews 2012) that show Y27 to block mitotic rounding to a similar extent to RhoA inhibition. To firm up this conclusion, the authors should show that RhoA activation (eg using CN03) directly increases pERM levels in IF or western blot. It would also be great to compare with p-Myosin II in this context.

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We agree with Reviewer 2 that the relatively mild effect on mitotic cell rounding observed following ROCK inhibition may seem at odd with Maddox 2003 or Matthews 2012 at first sight.

Maddox 2003 showed that both RhoA and ROCK inhibitions affect rounding of mitotic cells. However, the purpose of this article was not to compare the effect of RhoA with the effect of ROCK inhibition. Indeed, results presented in Figures 1 and 2 of this paper suggest that RhoA inhibition has a greater inhibition on cell rounding than ROCK inhibition ($\approx$ +80% cell area after RhoA inhibition, $\approx$ +25% cell area after ROCK inhibition). This is in accordance with what we are reporting in our manuscript (Fig 3L vs 4I)

Matthews 2012 measured the timing of rounding but did not quantify the effect of RhoA or ROCK inhibition on cell sphericity in metaphase *per se*. Using phase contrast time-lapse microscopy, they showed that both RhoA and ROCK inhibitions led to a delay in mitotic rounding. However, unlike 3D reconstruction after confocal microscopy, which we used in our manuscript, phase contrast microscopy does not allow to quantify sphericity parameters. This makes the comparison between our respective results difficult.

Finally, as suggested by Reviewer 2, we agree that showing that RhoA activation triggers ERM and MLC2 phosphorylation in HEK293T would be a great addition to our manuscript. The attached Fig. R5 shows that CN03-induced RhoA activation triggers a significant increase of both p-ERM and p-MLC2 levels in HEK293T cells. This new figure (R5) will replace and complement Fig S2C-D that was showing the effect of Y-27632 on p-MLC2.

Revision Plan
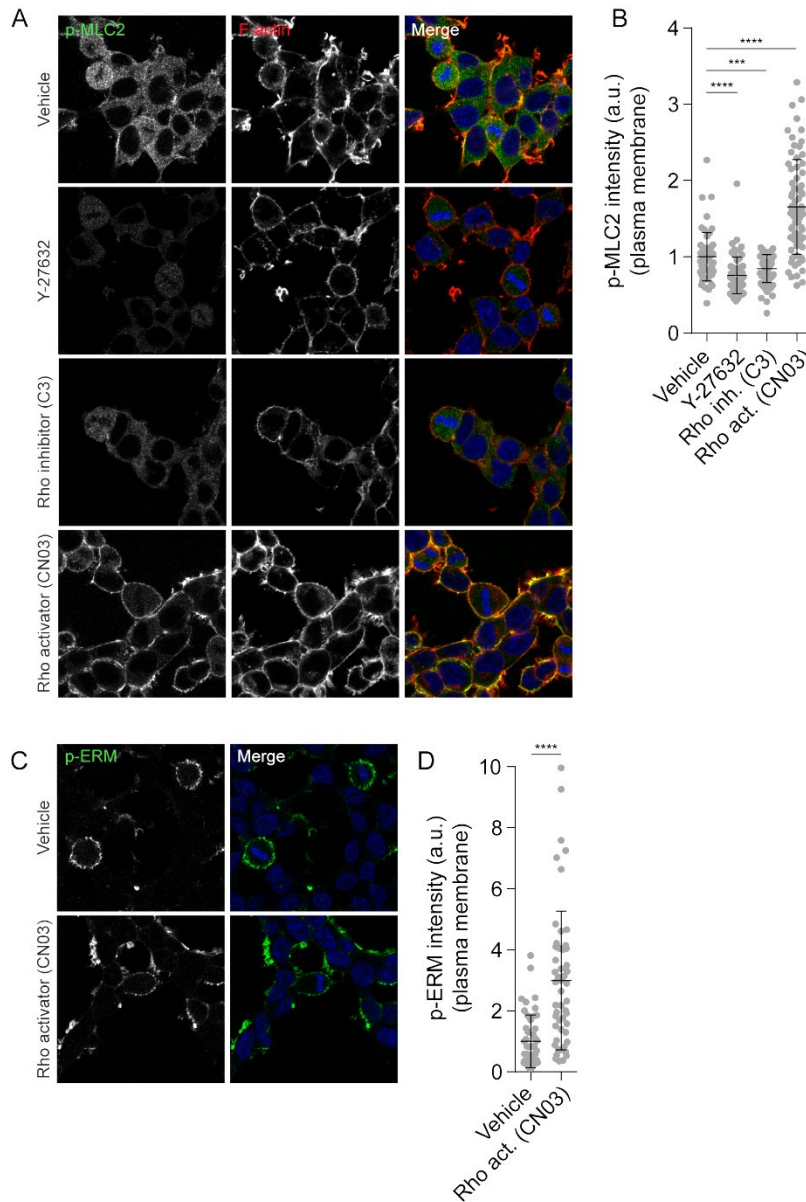
Figure R5: CN03-induced RhoA activation triggers both MLC2 and ERM phosphorylation in HEK293T cells.

(A-B) p-MLC2 (Ser19) and F-actin staining of HEK293T cells incubated with vehicle, ROCK inhibitor Y-27632 (10 µM, 4h), Rho inhibitor C3 (1 µg/mL, 6h) or Rho activator CN03 (1 µg/mL, 2h).
(C-D) p-ERM staining of HEK293T cells incubated with vehicle or Rho activator CN03 (1 µg/mL, 2h).
Immunofluorescences are representative of three independent experiments. P-MLC2 and p-ERM quantifications represent the mean +/- s.d. of three independent experiments. Dots represent individual cells. P values were calculated using a two-tailed t test. ***, P < 0.001. ****, P < 0.0001.

Reviewer 3-1. Can an effect of taxol on MTs be detected? The spindles shown look similar with or without taxol. Are there delays in the disassembly of interphase MT arrays that could explain the effect of taxol? Additional data could help clarify whether the effect of taxol is consistent with the authors' interpretations.

See answer to Reviewer 1-3 who had the same concern.

Reviewer 3-2.

Revision Plan

See section 4 of this revision plan.

[Minor comments of Reviewers](#)

We will add to the revised manuscript quantifications of the western blots (Fig 1E-F) and immunofluorescences (Fig 1G) that are missing, as well as some modifications/additions in the text and figures asked by the reviewers.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Please insert a point-by-point reply describing the revisions that were <u>already carried out and included</u> in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

4. Description of analyses that authors prefer not to carry out

Please include a point-by-point response explaining why some of the requested data or additional analyses <u>might not be necessary or cannot be provided within the scope of a revision</u>. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

Reviewer 3-2. Mitotic rounding can involve focal adhesion disassembly, and a reduction of attachment to the substrate, presumably reflected by the authors' assays of cell X-Z dimension ratios in side views (the sphericity assays). The Bershadsky lab recently showed that GEF-H1 seems to be specifically regulated by KANK-based MT attachment to focal adhesions (PMID: 31114072). For example, KANK depletion resulted in GEF-H1 activation without loss of the full MT network. The current work could be further fleshed out by investigating whether depletion of KANK increases sphericity and ERM phosphorylation in interphase cells. Is it the general remodeling of the MT cytoskeleton or the specific loss of MT-FA attachments that are important? Experimental investigation of this point may or may not go beyond the scope of the current paper, but the KANK-based mechanism of GEF-H1 regulation should be discussed at minimum. Also, the cell culture substrate should be added to the Methods section.

We thank Reviewer 3 for this important suggestion. However, as mentioned by Reviewer 2 in the "referee cross-commenting" section and stated by Reviewer 3, this new aspect appears beyond the scope of our current work. As suggested, we will discuss the KANK-based mechanism of GEF-H1 regulation in the revised version of our manuscript.

October 11, 2021

Re: JCB manuscript #202109065T

Prof. Sébastien Carréno
University of Montreal
Pathology and Cell Biology
Marcelle-Coutu Pavilion - room 3306-5 C.P. 6128 Succursale Centre-Ville
Montreal, Quebec H3C3J7
Canada

Dear Prof. Carréno,

Thank you for submitting your manuscript entitled "Interphase microtubule disassembly is a signaling cue that drives cell rounding at mitotic entry". We apologize for the delay in communicating a decision to you. We have assessed your manuscript, the reviewer reports, and your revision plan. We feel that your work is intriguing and will likely be of interest to cell biologists. Therefore we would like to invite you to submit a revision if you can address the reviewers' key concerns. We have also evaluated the suitability of your revision plan and such plan seems reasonable to us.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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 an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Transfers may have up to 10 main text figures. 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.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Transfers may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

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.

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.

Sincerely,

Karen Oegema
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD

Scientific Editor
Journal of Cell Biology

Point-by-point response to the reviewer comments:

We are pleased that the three reviewers found that our work is *"an elegant and well-presented study with convincing results that justify the conclusions"* and that they acknowledged that *"these findings will be relevant to an audience interested in understanding the molecular mechanisms underlying morphological changes of mitotic cells"* and *"to cell biologists interested in mitotic rounding as well as those more generally interested in cell division, the acto-myosin cytoskeleton and Rho GTPases"*. We would like to thank the 3 reviewers that made a very thorough and constructive assessment of our manuscript. We have taken in consideration every of their suggestions and we feel that it helped improving our manuscript. We are happy to provide new experiments and new quantifications that further demonstrate the role of microtubule depolymerization in ERM activation and cell rounding during mitosis.

Reviewer #1:

We thank the reviewer who qualified our work *"thorough and clearly presented"* and found that *"the evidence brought forward convincingly fits the proposed model"*. We also thank the reviewer for his/her constructive comments.

1.1. The authors quantify the ratio between cell height and width (which they define as sphericity) as a measure for cell rounding, but this approach has several concerns. It is not clear how cell width is determined and the performed measurements will be greatly determined by which cell axis is used to measure this (and this appears to be the length across the arbitrary x-axis).

We agree with Reviewer 1 that measurement of cell sphericity can be affected by the x-axis chosen. We apologize for our lack of precision because we did take this concern into consideration when we determined cell width to calculate sphericity. Indeed, cell width was calculated as the average of the longer axis of the cell and its orthogonal in order to not be biased by the orientation of cells in the x/y plane. For more clarity, we included a diagram in Fig 3G as well as the following section in the material and methods section of the revised manuscript: *"For cell sphericity measurements, orthogonal views were extracted using Zen software (Zeiss). On these views, several parameters were measured using ImageJ software (NIH): cell height (z) and cell width determined as the average between the longer axis (L) and its orthogonal (O). Cell sphericity was then determined as a ratio between cell height and cell width."*

1.2. To better understand the shown phenotype, alternative measures for cell rounding that are more conventionally used should be included, at least showing cell height also separately from the cellular shape within the xy plane (the latter can be either cell area/length of short or long axis/perimeter/circularity).

We thank Reviewer 1 for these suggestions, and we agree that these alternative measures will provide a better description of the shown phenotypes. We therefore included in the supplementary data of the revised manuscript cell height (z) and cell shape within the x/y plane (measured as the average length of the longer axis (L) and its orthogonal (O)) for all quantifications of cell sphericity presented in the initial manuscript (Fig S3, S4, S5).

1.3. Furthermore, in order to truly be able to interpret the data on defects in mitotic cell rounding, the authors should compare mitotic cell shape with the shape of the same cell in interphase prior to entering mitosis (which can be easily done in live-cell experiments, with for instance membrane markers).

We agree with Reviewer 1 and Reviewer 2 (in the referee cross-commenting section) that a better description of interphase cells compared to metaphase cells would help to better assess how cells round-up in metaphase and if cell rounding is truly affected after any given treatment/RNAi. To address this, we now present for each

quantification of cell shape (sphericity, cell height, cell shape within the x/y plane) side to side measurements of interphase (I) and metaphase (M) cells (see Fig 5B,F,J, Fig 6B,E,H,K, Fig 7C,H,K, Fig S3A-F, Fig S4A-D,F-H,J-M, Fig S5C-D,G-H,J-K). While Reviewer 1 suggested to perform live-cell experiments, we chose to characterize interphase cells in fixed samples since this method allows measurement of a larger number of cells than with time-lapse experiments. Therefore, we believe that the presented data are more quantitative.

In addition, we quantified cell shape transformation from interphase to metaphase by calculating the sphericity ratio of metaphase to interphase cell populations in at least three independent experiments. We believe that this representation better quantifies cell rounding from the interphase cell population to the metaphase cell population (see Fig 5C,G, Fig 6C,F,I, Fig 7D,I Fig S4I, Fig S5L).

2.1. The model proposed in this manuscript to a great extent depends on pre-existing knowledge from literature (e.g. GEF-H1 and Rho activation following microtubule disassembly, ref 20 and 21; nocodazole-induced cell rounding through GEF-H1, ref 20; Rho-mediated Slk activation, ref 18; Slk/ERM-dependent mitotic cell rounding, ref 2,3). While this is on itself is not problematic, several key components of their model should also be experimentally tested in their cellular system. Most importantly, the authors propose that SLK depletion disturbs mitotic rounding through the absence of ERM activation; but although the role of ERM proteins in mitotic rounding has been demonstrated in Drosophila cells, mammalian cells depleted of ERM proteins were previously shown not to have any defect in mitotic cell rounding (Machicoane et al., 2014). The authors should therefore perform experiments with ERM depletion and phospho-dead/mimetic mutants to test whether SLK truly functions through ERM in mitotic rounding in HEK293T cells.

We agree with the reviewer that while the roles of ERM proteins and SLK in rounding of mitotic cells have been clearly established in drosophila (dMoesin and Slik), these roles are less clear in mammalian cells. Indeed, Machicoane and colleagues (2014) reported that mammalian HeLa cells depleted for SLK or all three ERMs did not display lack of cell rounding (PMID: 24958772). Accordingly, movies of SLK or ERM RNAi cells shown in this article showed that metaphase cells appear to round up. However, this was observed by phase contrast time-lapse microscopy, a technic that cannot quantitatively measure the 3D shape of mitotic cells. In contrast, Toyoda and colleagues (2017) showed that ezrin RNAi silencing led to a decrease of metaphase cortical tension in HeLa cells (PMID: 29097687). Since increase of cortical tension at mitosis onset provides an essential force that prompts metaphase cell rounding, their data suggest that the role of ERM proteins for metaphase rounding is conserved in mammalian cells.

Thus, to support our conclusions and as proposed by the reviewer, we assessed the role of ERMs in metaphase HEK293T cell rounding using 3D reconstruction after confocal microscopy (Fig 5). We found that depletion of SLK or co-depletion of all three ERMs using RNAi led to a significant decrease of metaphase cell sphericity, as well as a decrease of cell sphericity ratio between interphase and metaphase populations. These results support our conclusions that ERM are involved in metaphase cell rounding in mammalian cells.

Furthermore, to strengthen our model and as proposed by the reviewer, we included in the revised manuscript rescue experiments using an ezrin phosphomimetic mutant (ezrin[T567D]) overexpressed in SLK depleted cells (Fig 5H-J). We found that ezrin[T567D] rescued metaphase cell rounding defects caused by the depletion of SLK, supporting our conclusion that SLK controls mitotic rounding through ERM activation. Importantly, expression of a non-phosphorylatable mutant, ezrin[T567A], did not rescue SLK depletion and promoted by itself cell rounding defects in control cells. These observations are in fact consistent with this mutant behaving as a dominant negative mutant (Parnell, BBA 2015).

2.2. Similarly, the authors should show that disruption of microtubules indeed leads to activation of Rho in these cells.

While disruption of microtubules has already been shown to activate RhoA in several cell types (Lorenowicz, Am J Physiol Lung Cell Mol Physiol 2007; Chang, MBC 2008; Hiyoshi, Plos Pathogens 2015), we agree that this observation has never been made in HEK293T cells. To address this issue, we included in the revised manuscript a rhotekin-RBD pull down assay (now in Fig 2A,B). Using this approach, we showed that nocodazole treatment leads to an increase of GTP-bound RhoA, in agreement with a role of microtubule disassembly in activation of RhoA in HEK293T cells.

3. In Fig. 3, the authors use low levels of Taxol to prevent interphase microtubules disassembly and to test whether this prevents ERM activation and mitotic cell rounding. These experiments lack controls that interphase microtubule disassembly is truly inhibited, which should be included. Furthermore, as stated by the authors "interphase microtubule disassembly frees tubulin dimers that can then re-polymerize to form the mitotic spindle", and it is therefore unclear how this taxol treatment would still allow formation of the mitotic spindle (Fig 3a).

We agree with the reviewers 1 and 3 (see point 1 for reviewer 3) that if disassembly of interphase microtubules is perturbed by low doses of taxol, it should lower the availability of tubulin dimers and thereby affects to some extent the formation of the mitotic spindle.

We used the same low concentrations of taxol to perturb the disassembly of interphase microtubules at mitosis entry than Mchedlishvili and colleagues (2018). They already have intensively characterized this treatment in HeLa cells in their article. However, we did not mention in our initial manuscript if low doses of taxol affected mitosis in HEK293T cells and we apologize for this omission. To address this issue, we included in the revised manuscript quantifications of the mitotic index and characterization of the mitotic spindle (now in Fig S2F-H of the revised manuscript). In this figure, we showed that low of taxol (10 nM) increased the number of mitotic cells compared to control indicating a slower mitosis. In addition, we measured an increase of monopolar spindles in metaphase demonstrating that the effect of low doses of taxol is consistent with our interpretations, since as one could expect, it affected the formation of the bipolar mitotic spindle.

4. For some WBs, quantifications are lacking. This is especially important to include because the total ERM levels also seem to be affected by microtubule targeting drugs (e.g. 1E)

Quantifications of all western blots have been included in the revised manuscript.

5. Knockdown efficiencies for siRNA SLK #1 and SLK #2 (3MN, 4HI) are not shown (only for shRNA)

Western blot showing knock-down efficiencies for siRNA against SLK that was missing in the initial manuscript are now included in the revised manuscript (Fig 3C).

6. The representation of data can sometimes be improved: several representative IF images are relatively small, making it difficult to see the entire contour of the cell (e.g. 4F); individual channels are sometimes not properly visible in merged images, which for instance makes it is difficult to see the effect of nocodozole on tubulin in 2J; and some of the shown examples do not clearly represent the quantified data.

We made several changes to improve our figures. For instance, we underlined the periphery of the cells using dashed white lanes in the orthogonal views.

7. Introduction and discussion are lacking

The revised manuscript is now written under an article format, including an introduction and a discussion.

Reviewer #2:

We thank the reviewer who qualified our work as *"an elegant and well-presented study with convincing results that justify the conclusions".* We also thank the reviewer for his/her constructive comments.

1. The most worrying point is that the authors appear to switch between using siRNA and shRNA knockdown for SLK and GEF-H1. The authors don't draw attention to this in the text or mention why different methods are used. For example, for SLK: shRNA is used in figure 2A, siRNA in figure 2J-K, shRNA in fig 3I-J and siRNA in figure 3M-N and then siRNA in figure 4H. For figure 3, the converse experiments are presented in the supplement but not for the others. Worryingly, where both sets are included, the results are slightly different: in Fig 3J, the increase in pERM from interphase to mitosis is not significant with shRNA, while in Fig S3D, using siRNA, it is. Although this difference doesn't change the overall conclusions, it makes it look as though the authors have tried both methods and picked the one with the most convincing result for the main paper. In addition, while the shRNA knockdown is validated by the blot in figure 2A, the siRNA knockdown is not. If the authors want to use two different methods to deplete SLK, both need to be validated by western blot and where possible, the data from both methods shown in the supplement. The authors also need to make it clear in the text where they are switching methods and why.

We apologize for this lack of clarity. We routinely used both siRNA and shRNA to perform our knock-down experiments. We believe that this approach is one of the best approach to avoid that the phenotypes we study are due to off-target effects. Yet, we agree that switching between siRNA and shRNA may appear confusing even if, as stated by reviewer 2, it does not change our overall conclusions. For more clarity, we now indicate in the main text where and why we are using siRNA or shRNA.

Finally, as mentioned before in response to Reviewer 1 (see point 5), western blot showing knock-down efficiencies for siRNA against SLK has been included in the revised manuscript (see Fig 3C).

2. Much of the conclusions in figures 3 and 4 come from measurements of pERM levels at the plasma membrane in mitosis, however there is not enough information given about how these measurements are made. How was the plasma membrane identified (especially in interphase conditions where pERM levels are very low)? Was intensity measured all around the membrane or just at certain points? Was it the averaged per cell? It's stated that 'p-ERM signal intensity at the plasma membrane is normalized to interphase cells treated with vehicle'. Does this mean that they are normalising across different conditions? It's hard to ensure identical imaging conditions for different samples, so was any kind of internal normalisation also applied? Eg. to background or cytoplasmic levels?

We agree with Reviewer 2 that some information was missing in our initial manuscript concerning how p-ERM levels were measured at the plasma membrane. As stated in our initial revision plan, the following information has been added to the material and methods section of the revised manuscript: *For quantifications of p-ERM levels, images were acquired with identical microscope settings across the same experiment. p-ERM signals were measured all around the plasma membrane using the segmented line tool from Image J software (NIH). p-ERM intensity is represented as the average of p-ERM levels at the plasma membrane per cell, in which background was subtracted. In experiments were p-ERM levels were quantified in both interphase and metaphase cells, p-ERM levels per cell were normalized to the average of p-ERM levels in interphase control cells (vehicle or si/shRNA non-target). Thus, the average of normalized p-ERM levels in control interphase cells is equal to 1.*

In addition, as stated in the initial revision plan, we routinely assess the background signals from each condition that should be comparable (see Fig R1 of this point-by-point response).
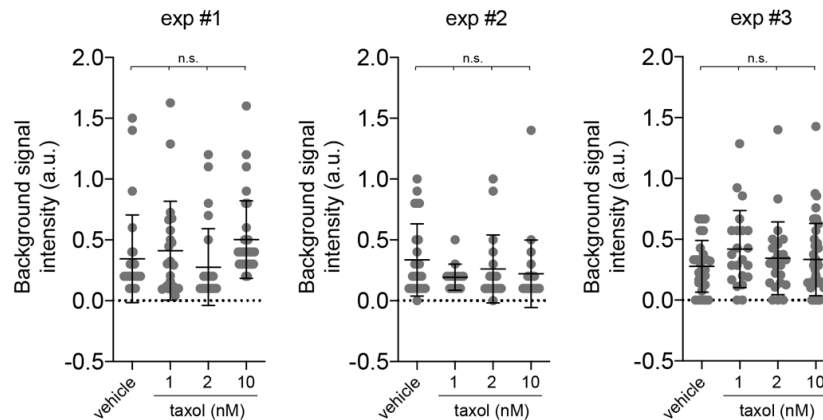
Figure R1: Background signal intensity measured by immunofluorescence.
Background signal intensity from three independent experiments (exp #1-3). HEK293T cells were treated with vehicle or taxol for 90 min before fixation and immuno-stained with p-ERM antibody (see Fig 4A-B of the revised manuscript). P values were calculated using a two-tailed t test. n.s., not significant.

3. This work identifies microtubule disassembly and GEF-H1 as triggers for mitotic rounding but proposes that they ultimately work by activating RhoA (alongside Ect2). In the model and text the authors apply that the main function of RhoA is to activate SLK and ERMs rather than ROCK/myosin. This is based on figure 4H-I, where they show that SLK depletion has a greater effect on mitotic cell shape than ROCK inhibition and that there is no synergy between them. The relatively mild effect of ROCK inhibition is at odds with previous studies in different cell types (eg Maddox 2003, Matthews 2012) that show Y27 to block mitotic rounding to a similar extent to RhoA inhibition. To firm up this conclusion, the authors should show that RhoA activation (eg using CN03) directly increases pERM levels in IF or western blot. It would also be great to compare with p-Myosin II in this context.

We apologize if some of our writing was misleading. We did not want to imply that the main function of RhoA is to activate SLK and ERMs rather than ROCK and myosin phosphorylation. Indeed, we even concluded in the manuscript that *"GEF-H1 and Ect2 both engage RhoA that relays the cell-cycle signals to its direct effectors, ROCK kinases and SLK. ROCK kinases take in charge the generation of actomyosin forces (Maddox and Burridge, 2003; Matthews et al., 2012) while SLK activates ERMs that link these forces to the plasma membrane (Fig 8). This integrated signaling network coordinates the generation of actomyosin forces with their coupling to the plasma membrane to drive cell rounding at mitotic entry."*

However, we realized that our model drawn in the initial manuscript could be misleading. The dashed arrows from RhoA to ROCK to myosin light chain 2 may implied that ROCK is less important than SLK-ERM for mitotic rounding. That was not our intention; we chose the dashed arrows because this ROCK pathway has been characterized before our manuscript. For more clarity, we therefore changed these dashed arrows for plain arrows in the model of the revised manuscript (now in Fig 8).

Following reviewer 2 suggestion, we now provide evidence that activation of RhoA using CN03 promotes the phosphorylation of ERMs and myosin light chain II in control cells (Fig S2A-B and S2K-L).

4. Figure 1G is not quantified. Also, the images are very small and the zoom is confusing - it's not clear what we are looking at without the merge.

Quantification of the immunofluorescence of p-ERM staining after nocodazole (Fig 1I) or podophyllotoxin (Fig S1I) have been added in the revised manuscript. Images have also been adjusted for more clarity.

5. Figure 2G: the authors state that GEF-H1 depletion blocks Nz-induced ERM phosphorylation. From their blots there is still an increase, it is just smaller. They should change the text to state that it 'decreases' the effect.

We agree with Reviewer 2 and we therefore changed the text accordingly in the revised manuscript as follows: "*Depletion of GEF-H1 using independent shRNA or siRNA decreased ERM phosphorylation after nocodazole treatment (Fig 2E-F and S2C-D)*".

Reviewer #3:

We thank the reviewer who acknowledged that our *"study's conclusions are supported by a variety of approaches, tests of alternate mechanisms, appropriate controls, and thorough quantifications and statistical analyses".* We also thank the reviewer for his/her constructive comments.

1. Can an effect of taxol on MTs be detected? The spindles shown look similar with or without taxol. Are there delays in the disassembly of interphase MT arrays that could explain the effect of taxol? Additional data could help clarify whether the effect of taxol is consistent with the authors' interpretations.

We agree with the reviewers 3 and 1 (see point 3 for reviewer 1) that if disassembly of interphase microtubules is perturbed by low doses of taxol, it should lower the availability of tubulin dimers and thereby affects to some extent the formation of the mitotic spindle.

We used the same low concentrations of taxol to perturb the disassembly of interphase microtubules at mitosis entry than Mchedlishvili and colleagues (2018). They already have intensively characterized this treatment in HeLa cells in their article. However, we did not mention in our initial manuscript if low doses of taxol affected mitosis in HEK293T cells and we apologize for this omission. To address this issue, we included in the revised manuscript quantifications of the mitotic index and characterization of the mitotic spindle (now in Fig S2F-H of the revised manuscript). In this figure, we showed that low of taxol (10 nM) increased the number of mitotic cells compared to control indicating a slower mitosis. In addition, we measured an increase of monopolar spindles in metaphase demonstrating that the effect of low doses of taxol is consistent with our interpretations, since as one could expect, it affected the formation of the bipolar mitotic spindle.

2.1. Mitotic rounding can involve focal adhesion disassembly, and a reduction of attachment to the substrate, presumably reflected by the authors' assays of cell X-Z dimension ratios in side views (the sphericity assays). The Bershadsky lab recently showed that GEF-H1 seems to be specifically regulated by KANK-based MT attachment to focal adhesions (PMID: 31114072). For example, KANK depletion resulted in GEF-H1 activation without loss of the full MT network. The current work could be further fleshed out by investigating whether depletion of KANK increases sphericity and ERM phosphorylation in interphase cells. Is it the general remodeling of the MT cytoskeleton or the specific loss of MT-FA attachments that are important? Experimental investigation of this point may or may not go beyond the scope of the current paper, but the KANK-based mechanism of GEF-H1 regulation should be discussed at minimum.

We thank Reviewer 3 for this important suggestion. However, and as mentioned by Reviewer 2 in the referee cross-commenting section and stated by Reviewer 3 him-/herself, this new aspect appears beyond the scope of our current work. As suggested by Reviewer 3, we now discuss the KANK-based mechanism of GEF-H1 regulation in the last paragraph of the discussion section of the revised version of our manuscript.

2.2. Also, the cell culture substrate should be added to the Methods section.

Cells were cultivated on regular cell culture flasks and plated on regular glass coverslips for immunofluorescence, without addition of any substrate. References for consumables have been added in the material and methods section.

March 4, 2022

Re: JCB manuscript #202109065R

Prof. Sébastien Carréno
University of Montreal
Pathology and Cell Biology
Marcelle-Coutu Pavilion - room 3306-5 C.P. 6128 Succursale Centre-Ville
Montreal, Quebec H3C3J7
Canada

Dear Prof. Carréno,

Thank you for submitting your revised manuscript entitled "Interphase microtubule disassembly is a signaling cue that drives cell rounding at mitotic entry". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

Reviewers #1 and #3 are supportive of publication pending that you address some remaining minor concerns, which can be done via text edits. Reviewer #2 notes that a requested experimental control in their original comments, which is showing that low levels of taxol indeed inhibit interphase MT disassembly, is missing. We agree with the reviewer that this control is important to demonstrate that MT disassembly at the transition between interphase and mitosis acts as cell-cycle cue controling ERM activation. This reviewer also requests to exclude a potential effect of taxol in cell rounding later in mitosis. We hope you can address the reviewer #2's points with new data and the rest of reviewers' concerns.

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.

Sincerely,

Karen Oegema
Monitoring Editor
Journal of Cell Biology

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

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

The authors have effectively addressed my past concerns. The revised paper is comprehensive, well-executed, and well-written. It advances the understanding of mitotic cell rounding with the discovery of an inductive mechanism, and by showing the relationship of the newly identified mechanisms with previously known mechanisms.

I have one remaining concern about this sentence: "The ratio of sphericity between the population of interphase and metaphase cells also decreased upon double depletion of Ect2 and GEF-H1 when compared to single depletions even if this reduction did not reach statistical significance (Fig 7I)." The conclusion about the decrease should not be made if the statistical test did not support the conclusion.

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

The authors have included additional experiments and analyses in their revised manuscript to support their conclusions. With these revisions they addressed most of my initial concerns.

I have one remaining concern that the authors did not fully address, regarding a key experiment using low levels of taxol to demonstrate that interphase microtubule disassembly at the transition between interphase and mitosis acts as cell-cycle cue that controls ERM activation. As indicated in my original comments, it is essential to include control experiments to show that in their experiments interphase microtubule disassembly is truly inhibited under these conditions. Because this is a key experiment to support their conclusion, only analyzing eventual spindle morphology and referring to previous work that was performed in another cell type does not suffice. Furthermore, I'm not fully convinced that from these experiments it can be concluded that a lack of disassembly of interphase microtubules is causing the downregulation of cortical pERM and impaired mitotic cell rounding after taxol treatment. For this, it is important to exclude a potential effect of taxol later in mitosis; i.e. showing that addition of these low levels of taxol after cells have entered mitosis (after interphase microtubule disassembly) does not affect cell rounding.

Minor comment:
The authors analyze cell shape within the x/y plane as average length of the long and orthogonal axis, which serves as a measure for cell spreading. This parameter does not show the lack of rounding of cells within the x/y plane with their various perturbations. Merely as a suggestion: the author may also include parameters such as circularity within the x/y plane, which would strengthen these analyses.

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

The authors have thoroughly and carefully addressed all the reviewers' points. As a result, they have significantly strengthened their conclusions and the manuscript quality. As mentioned in initial review, this work advances our understanding of the molecular mechanisms of cell rounding in mitosis and is likely to be of great interest to scientists working in this field as well as the wider community of cell biologists. Therefore, I strongly recommend publication of this work in JCB.

Minor points
Figure 3D: In the WB quantification pERM levels should be normalised to total ERM (not actin) as has been done for the other blots.
Discussion: It's not true to say that phase contrast imaging 'cannot quantitatively measure the degree of cell rounding'. It would be better to point out the difference in scales and accuracy between the two techniques

March 24, 2022

RE: JCB Manuscript #202109065RR

Prof. Sébastien Carréno
University of Montreal
Pathology and Cell Biology
Marcelle-Coutu Pavilion - room 3306-5 C.P. 6128 Succursale Centre-Ville
Montreal, Quebec H3C3J7
Canada

Dear Prof. Carréno:

Thank you for submitting your revised manuscript entitled "Interphase microtubule disassembly is a signaling cue that drives cell rounding at mitotic entry". 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.

*** Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. Please ensure that the particular red and green hues used in main figures 3H, 5A, 5E, 5I, 6A, 6D, 6G, 6J, 7G, 7J and supplemental figures 2A, 4C, 4I, 5B, 5I are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide micrographs for each of the 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.

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.).

*** 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 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.

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.

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.

*** 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.

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.

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. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the 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).

-- 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.

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

Sincerely,

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