Review COMMONS

JCB

Nuclear tension controls mitotic entry by regulating cyclin B1 nuclear translocation

Margarida Dantas, Andreia Oliveira, Paulo Aguiar, Helder Maiato, and Jorge Ferreira

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Revision 0

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this manuscript, Dantas and colleagues report that confinement is sufficient to restore G2/M transition in cells than can't adhere to their matrix. Exploring further the mechanisms involved, they show that confinement (dynamic cell compression) stimulates nuclear import of cyclin B1 and nuclear envelope permeability using cells in 2D culture. The authors observed that actomyosin contractility increases NE tension in cells preparing for prophase, leading to an increase in nuclear translocation of cyclin B1. However, a few inconsistencies between the data and the conclusion make the current report too preliminary for publication. It may require significant additional work to consolidate the authors' model.

- The specific contribution of Nuclear Envelope tension. The authors conclude that confinement acts through increasing NE tension, although confinement may affect cytoplasmic signaling, which could contribute to G2/M transition. The authors should test whether compressing the nucleus versus compressing the cytoplasm have distinct effects on cyclin B1 nuclear translocation and G2/M, as it has been done by others when addressing nuclear mechanosensitive mechanisms (Elosegui-Artola et al. or Lomakin et al.). To consolidate their model, the authors should also test whether decreasing NE tension (independently of actomyosin tension) has opposite effect on G2/M (for example using LBR overexpression). Increase in nuclear membrane tension has been shown to trigger cPLA2 recruitment to the NE (Enyeidi et al, 2013; Lomakin et al. 2020), although the authors show here that confinement does not induce cPLA2 recruitment (but still increases NE tension figure 4G) in the absence of Rock activity or when the LINC complex is disrupted. This is surprising considering that confinement should increase NE tension independently of actomyosin contractility and should increase cPLA2 recruitment at the NE, unless in this case cPLA2 recruitment is not mediated by an increase in NE tension.
- NPC transport versus NE permeability. The authors suggest that confinement increases cyclin B1 transport via NPC-mediated transport and rule out that confinement may affect NE permeability based on the absence of NE rupture using the INM marker lap2. However, the sample size for this observation is missing and NE permeability could be altered even in the absence of major INM rupture observed by confocal. The authors should use a reporter of nuclear permeability (fluorescent cytoplasmic marker or nuclear marker as previously used by Denais et al or, 2016 or Raab et al., 2016) to make sure that NE permeability is not affected by confinement. In addition, NPC function should be tested in parallel with other fluorescent reporter (such as NLS-GFP constructs) to test whether global NPC-mediated transport is changed during prophase (with or without confinement).
- Effect of confinement on cyclin B transport (NEP) in adherent cells. In figure 1D, we can see that confinement enhances cyclin B1 nuclear translocation in cells adhering on fibronectin. Although it is unclear whether confinement has a significant effect in other figures, for example

in figure 2F: DMSO is not significantly different from confiner+CDKi (same thing in 3i and 3j with Rock inhibitor and Kash construct). In these figures the untreated+confiner (or control in 3j) is missing, and the absence of difference between treated+confiner and control is puzzling. Either there is no difference between confiner and CDKi+confiner and it means there is no difference between control and confiner (surprising considering figure 1D); or there is a difference between CDKi+confiner and confiner, indicating that CDK inhibition affects confinement-induced cyclin B import. Both possibilities suggest that the authors should significantly revisit their model. In any case, all control (untreated, treated +/- confiner should be in all figures to avoid any misunderstanding).
- Consequences of cPLA2 recruitment at the NE. The authors state that "Active cPLA2 then stimulates actomyosin contractility creating a positive feedback loop" But the NE is already unfolded and distance between NPR is increased before cPLA2 recruitment. Does PLA2 inhibition affect nuclear irregularity (or distance between NPC)? Or does cPLA2 impact cyclin B1 transport via a distinct mechanism? Did the author analyze CDK1 phosphorylation in presence of PLA2 inhibitor?
- Robustness of the main observation. On page 4, the authors report that cells enter mitosis after 140 sec (+/- 80 sec) of confinement, although in the example showed in figure 1b, the cell enters at least 420 sec min after confinement, as we can see that the cell is already confined -420 sec (compressed shape) and NEP occurs at 0. Did the author showed a cell that was not included in their statistics? This would be very surprising considering the very low sample size used for this experiment (n=6 and 10). In addition, many observations have been made on small sample size (n=6 for figure 1) or/and not from independent experiments. The authors should increase their sample size and compare results from independent experiments to consolidate their model.
- 2h shows nuclear signal (cyclin in grayscale), while 2e does not, why?
- starting point to quantify cyclin entry is the lowest intensity, which may depend on many factors (and could be affected by experimental design). It would be necessary to have synchronized cells to homogenize the starting point of these experiments.
- DN-KASH have been transiently transfected for single cell experiments, how does the authors unsure that cell observed are transfected? Does it have a fluorescent tag, if so which one?
- "requires contact with external stimuli" or "that mechanical confinement is sufficient to overcome the lack of external stimuli." (page 4): external stimuli is vague here and it could be better to replace it with a more specific description

2. Significance:

Significance (Required)

While the physiological relevance of these findings remain to be determined, the authors report an interesting observation that could have a significant impact in the field.
The authors do not comment the potential overlap of their findings with other reports involving the LINC complex (Booth et al., ELife) or CDK-mediated actin remodeling (Ramanathan et al., NCB 2015) during prophase.

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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No

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

Dantas and colleagues use mechanical confinement assays to demonstrate that both mitotic entry and the timing of prophase are sensitive to mechanical perturbations. They identify a novel mechanism that fine tunes the dynamics of cyclin B1 nuclear import during prophase whereby acto-myosin contractility leads to nuclear membrane unfolding, cPLA2 recruitment and cyclinB1 nuclear import. They show how mechanical confinement can accelerate this mechanism by independently inducing nuclear unfolding, and that this can go on to induce defects in mitotic spindle assembly and chromosome segregation.

Major

This work contains an impressive amount of data including some technically challenging experiments. The conclusions are convincing and for the most part well supported by experimental evidence (for exceptions see below). Appropriate controls are presented and statistical analysis is adequate. The methods are mostly described well but some important details are omitted (see below). The methods and figure legends would benefit from expansion, particularly in describing how the images presented relate to quantification in graphs. Although generally the manuscript is well written, there are parts when both the experimental logic and

conclusions are hard to follow, particularly in the description of figures 1 and 5 (see below for details). With a large amount of data, including important experiments relegated to supplementary figures, this work would benefit from expansion into a longer article format to allow for more clarity. Particularly:

- Figure 1A-C: here the authors show that non-adherent cells only enter mitosis when confined. There is some key information lacking here, including the experimental timeframe. How long were the cells plated on pll-peg before imaging and for how long were they imaged? In 1C, 80% of confined cells enter mitosis, which implies that cells were filmed for a relatively long time (given an average cell cycle length of 20-24 hours). Unless of course cells were previously synchronised in G2 but the authors do not state that this is the case. In the legend it states that images were acquired every 20s. Imaging cells for 20+ hours every 20s with multiple zs is likely to have a very deleterious effect on cells and to disrupt mitotic entry itself. The authors need to explicitly explain the experimental set-up used to generate the graphs in figure 1. In 1C, it would also be good to see the equivalent adherent control included in the graph (ie % cells that enter mitosis on fibronectin in the same timeframe). The authors use the data in 1A-C to claim that 'the G2-M transition requires contact with external stimuli'. However they haven't shown this, only that non adherent cells don't enter mitosis. To show that the G2/M transition is affected, they need to look at the cell cycle phase of cells on PLL-PEG and show that cells become arrested specifically in G2.
- Figure 5: The explanation of the conclusions here was hard to follow. It's not immediately clear why a faster prophase would lead to chromosome attachment delays in metaphase or segregation errors in anaphase since these events occur only after NEP. I think the authors' hypothesis is that a faster prophase results in less time for centrosome separation and that this is responsible for later spindle defects but this is not very clearly stated. If this is the case, then one might expect cells in which centrosome separation is delayed to also be the cells with lagging chromosomes. Did the authors observe such a correlation? It's also not clear why the authors expected confinement to rescue the spindle defects imposed by STLC treatment (supp figure 5). An alternative hypothesis that the authors neglect to mention is that faster cyclinB1 entry into the nucleus could also induce defects through changes to nuclear events such as chromosome condensation? Did they also see any changes to the rate of chromosome condensation in the confined prophase? Either way, the authors should explain more clearly in the text what they think is happening here.

Minor

- No reference is cited for the endogenous tagged CyclinB1 RPE1 line nor are any details about its construction given. Has this cell line been previously published by the Pines lab? Are one or both alleles tagged? N or C terminus?
- Figure 1C: presumably n in this case is number of experiments, not cells. How many cells were analysed in each case?
- Figure 1H. Why do the graphs have different scales on the x axis? Where does 101+-12s for confined cyclin B translocation mentioned in the text come from? From the graph, it looks longer than this?
- Figure 3 J, K. Confinement is able to rescue the effect of Y27 on cyclin B dynamics but not shROCK1. Why this difference? The authors should discuss this discrepancy in the text.

2. Significance:

Significance (Required)

This work identifies a novel mechanical mechanism that regulates the timing of cyclin B1 nuclear import in early mitosis. The role of nuclear unfolding in controlling cyclinB1 import is particularly interesting. How important this new mechanism will be in controlling the duration of prophase or mitotic fidelity in a 'normal' mitosis within a tissue is not yet clear. However, it raises many intriguing questions about how cells' mechanical environment could impact mitotic entry, which could be relevant to disease situations where mechanics is altered such as fibrosis or cancer. The work is likely to be of interest to a wide range of cell and molecular biologists including those interested in cell cycle, mitosis, mechano-biology and nuclear biology.

I am a cell biologist working on mitosis and the cell cycle.

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

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Yes

Revision Plan

Manuscript number: RC-2022-01337R
Corresponding author(s): Jorge G. Ferreira

[The "revision plan" should delineate the revisions that authors intend to carry out in response to the points raised by the referees. It also provides the authors with the opportunity to explain their view of the paper and of the referee reports.

The document is important for the editors of affiliate journals when they make a first decision on the transferred manuscript. It will also be useful to readers of the reprint and help them to obtain a balanced view of the paper.

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1. General Statements [optional]

In this manuscript, we describe a new, mechanosensitive regulatory mechanism that fine tunes cyclin B1 nuclear translocation to control mitotic entry. We are grateful to the reviewers for their comments and suggestions and hope the proposed revision plan addresses all concerns raised. We are pleased that both reviewers recognize the potential impact for a wide audience and significance of the work presented in this manuscript.

2. Description of the planned revisions

Reviewer 1

"To consolidate their model, the authors should also test whether decreasing NE tension (independently of actomyosin tension) has opposite effect on G2/M (for example using LBR overexpression)".
This is a relevant point raised by the reviewer. We will overexpress LBR-RFP in our RPE-1 cyclin B1-Venus cell line and measure cyclin B1 nuclear translocation in comparison to unmanipulated cells. In addition, we will measure the levels of cPLA2 on the nuclear envelope upon LBR overexpression, to determine whether decreasing NE tension is reflected in a decreased cPLA2 recruitment.

"NPC transport versus NE permeability. The authors suggest that confinement increases cyclin B1 transport via NPC-mediated transport and rule out that confinement may affect NE

permeability based on the absence of NE rupture using the INM marker lap2. However, the sample size for this observation is missing and NE permeability could be altered even in the absence of major INM rupture observed by confocal. The authors should use a reporter of nuclear permeability (fluorescent cytoplasmic marker or nuclear marker as previously used by Denais et al or, 2016 or Raab et al., 2016) to make sure that NE permeability is not affected by confinement. In addition, NPC function should be tested in parallel with other fluorescent reporter (such as NLS-GFP constructs) to test whether global NPC-mediated transport is changed during prophase (with or without confinement)".

To address this, we propose the following: increase sample size for cells expressing lap2beta and clearly indicate it in the text, express NLS-GFP in RPE1-cells under confinement and measure its nucleocytoplasmic translocation and express cGAS-GFP in RPE-1 cells under confinement, to confirm that the nuclear envelope does not rupture.

"Effect of confinement on cyclin B transport (NEP) in adherent cells. In figure 1D, we can see that confinement enhances cyclin B1 nuclear translocation in cells adhering on fibronectin. Although it is unclear whether confinement has a significant effect in other figures, for example in figure 2F: DMSO is not significantly different from confiner+CDKi (same thing in 3i and 3j with Rock inhibitor and Kash construct). In these figures the untreated+confiner (or control in 3j) is missing, and the absence of difference between treated+confiner and control is puzzling". Either there is no difference between confiner and CDKi+confiner and it means there is no difference between control and confiner (surprising considering figure 1D); or there is a difference between CDKi+confiner and confiner, indicating that CDK inhibition affects confinement-induced cyclin B import. Both possibilities suggest that the authors should significantly revisit their model".

We thank the reviewer for pointing this out. However, we do not believe it is necessary to revisit our model based on this observation, for the reasons we now explain. We would not expect DMSO to be different from confiner+CDK1i. Given that CDK1i alone completely abolishes cyclin B1 transport due to lack of CDK1 activity (Gavet and Pines, 2010), we reasoned that the translocation of cyclin B1 observed in the confiner+CDK1i group is dependent on the mechanical stimulation. This is sufficient to restore cyclin B1 translocation, but not above control levels (fig. 2f). This situation is different from what is observed in figure 1, since these cells are confined in the presence of active CDK1. Therefore, in the conditions described in figure 1, we expect confiner to be different from controls (figure 1), as confinement is accelerating an already ongoing process. The same cannot be said for confinement in the presence of CDK1i, that restores cyclin B1 translocation previously blocked by CDK1 inhibition (Figures 2a-c and f). We propose to clarify the text to reflect these changes.

"In any case, all control (untreated, treated +/- confiner should be in all figures to avoid any misunderstanding)".

We will add a corresponding DMSO+confiner group to figures 2f, g and h. Similar groups will also be added to figure 3.

Revision Plan

“Consequences of cPLA2 recruitment at the NE. The authors state that "Active cPLA2 then stimulates actomyosin contractility creating a positive feedback loop" But the NE is already unfolded and distance between NPR is increased before cPLA2 recruitment. Does PLA2 inhibition affect nuclear irregularity (or distance between NPC)? Or does cPLA2 impact cyclin B1 transport via a distinct mechanism? Did the author analyze CDK1 phosphorylation in presence of PLA2 inhibitor”?

To address this, we propose to measure nuclear irregularity and NPC-NPC distance after cPLA2 inhibition. To confirm if cPLA2 might affect cyclin B1 through an alternative mechanism as the reviewers suggests, we propose to analyze CDK1 phosphorylation following cPLA2 inhibition. This will rule out any effect cPLA2 might have on CDK1 activity.

“Robustness of the main observation. On page 4, the authors report that cells enter mitosis after 140 sec (+/- 80 sec) of confinement, although in the example showed in figure 1b, the cell enters at least 420 sec min after confinement, as we can see that the cell is already confined -420 sec (compressed shape) and NEP occurs at 0. Did the author showed a cell that was not included in their statistics? This would be very surprising considering the very low sample size used for this experiment (n=6 and 10). In addition, many observations have been made on small sample size (n=6 for figure 1) or/and not from independent experiments. The authors should increase their sample size and compare results from independent experiments to consolidate their model”

Although this is not the main focus of the paper, we do agree that additional measurements are required. We will increase sample size for cells seeded in PEG with or without confinement (Fig. 1a-c). We will add this information to the corresponding figures.

“2h shows nuclear signal (cyclin in grayscale), while 2e does not, why”?

We do not understand what the reviewer means with this observation, since fig 2h is a graph and fig 2e shows nuclear cyclin in gray scale (right panels).

“starting point to quantify cyclin entry is the lowest intensity, which may depend on many factors (and could be affected by experimental design). It would be necessary to have synchronized cells to homogenize the starting point of these experiments”.

The majority of our cyclin B1 quantifications are normalized for the lowest value inside the nucleus. The reason for this is because prior to the increase in CDK1 activity in late G2, cyclin B1 nuclear levels are residual due to a predominance of nuclear export (Hagting et al., 1998; Toyoshima et al., 1998). Therefore, any increase in cyclin B1 nuclear import or decrease in its export should be readily observed. Moreover, we also clearly show that cyclin B1 translocation is accelerated upon confinement, even when we do not take into account cyclin B1 lowest intensity (Figure 1g and 1h).

Although synchronizing cells has been widely used in the past, we tend to avoid unnecessary manipulation of cells. In fact, protocols for cell synchronization in G2

involve either CDK1 inhibition (which is not recommended in this situation, as it directly affects the kinase controlling cyclin B1 translocation) or a double thymidine block which can cause DNA damage (Darzynkiewicz et al., 2011), known to delay cyclin B1 translocation (Toyoshima et al., 1998). Notwithstanding, we will synchronize RPE-1 cells expressing cyclin B1-Venus/tubulin-RFP with a double thymidine block and follow cyclin B1 translocation.

"DN-KASH have been transiently transfected for single cell experiments, how does the authors unsure that cell observed are transfected? Does it have a fluorescent tag, if so which one"?
The DN-KASH construct that was transfected into cells has an RFP tag, so that we could select cells that were indeed expressing DN-KASH. We will add a representative image of DN-KASH-RFP to the appropriate figure.

"requires contact with external stimuli" or "that mechanical confinement is sufficient to overcome the lack of external stimuli." (page 4): external stimuli is vague here and it could be better to replace it with a more specific description."
We will clarify this sentence in the text

"The authors do not comment the potential overlap of their findings with other reports involving the LINC complex (Booth et al., ELife) or CDK-mediated actin remodeling (Ramanathan et al., NCB 2015) during prophase".
We will comment and discuss our data relative to the papers cited by the reviewer.

Reviewer 2
"This work contains an impressive amount of data including some technically challenging experiments. The conclusions are convincing and for the most part well supported by experimental evidence (for exceptions see below). Appropriate controls are presented and statistical analysis is adequate. The methods are mostly described well but some important details are omitted (see below). The methods and figure legends would benefit from expansion, particularly in describing how the images presented relate to quantification in graphs".
We thank the reviewer for this comment. We will improve and clarify the methods and figure legends to link the images with the quantifications presented.

"Figure 1A-C: here the authors show that non-adherent cells only enter mitosis when confined. There is some key information lacking here, including the experimental timeframe. How long were the cells plated on pll-peg before imaging and for how long were they imaged? In 1C, 80% of confined cells enter mitosis, which implies that cells were filmed for a relatively long time (given an average cell cycle length of 20-24 hours)".
Along what was described for the previous point, we will clarify how this experiment was performed. Importantly, for 1C it does not mean that 80% of the entire population entered mitosis. Instead, 80% of the filmed cells entered mitosis following confinement. As we were filming with high spatial and temporal resolution, we had to resort to single cell imaging for every movie and for shorter periods of time. As a result, cells in PLL-g-PEG

only were filmed for a maximum of 3 hours. Cells on PLL-g-PEG+confiner were individually selected and confined, and entered mitosis shortly after (approximately 140 sec after confinement), as pointed out in the text.

"Unless of course cells were previously synchronised in G2 but the authors do not state that this is the case. In the legend it states that images were acquired every 20s. Imaging cells for 20+ hours every 20s with multiple zs is likely to have a very deleterious effect on cells and to disrupt mitotic entry itself. The authors need to explicitly explain the experimental set-up used to generate the graphs in figure 1".
As stated above in response to reviewer 1, we did not synchronize cells in G2. Because we were selecting cells that were in late G2 (as determined by cytoplasmic cyclin B1 levels and centrosome separation), it was possible to perform 3D, high temporal resolution imaging. In addition, confined cells were filmed for a maximum of 30 min, as the confinement triggered a fast mitotic entry. We will clarify the experimental setup used in figure 1.

"In 1C, it would also be good to see the equivalent adherent control included in the graph (ie % cells that enter mitosis on fibronectin in the same timeframe)".
We will quantify the % of cells seeded on fibronectin that enter mitosis and add this data to figure 1C.

"The authors use the data in 1A-C to claim that 'the G2-M transition requires contact with external stimuli'. However they haven't shown this, only that non adherent cells don't enter mitosis. To show that the G2/M transition is affected, they need to look at the cell cycle phase of cells on PLL-PEG and show that cells become arrested specifically in G2".
We agree that our data does not show a specific G2 arrest, as we did not look at cell cycle progression. In fact, we believe this to be out of the scope of this paper. According to the reviewer´s comment, we propose to rewrite the text to state that non-adherent cells fail to enter mitosis.

"*Figure 5: The explanation of the conclusions here was hard to follow. It's not immediately clear why a faster prophase would lead to chromosome attachment delays in metaphase or segregation errors in anaphase since these events occur only after NEP. I think the authors' hypothesis is that a faster prophase results in less time for centrosome separation and that this is responsible for later spindle defects but this is not very clearly stated. If this is the case, then one might expect cells in which centrosome separation is delayed to also be the cells with lagging chromosomes. Did the authors observe such a correlation"?*
We apologize for the lack of clarity. As the reviewer rightly points out, faster prophase means that NEP occurs before centrosomes have the time to properly separate and assemble a spindle. This has been previously shown by us and others to increase chromosome missegregation (Nunes et al., 2020; Silkworth et al., 2012; Kaseda et al., 2012). In fact, in one of our previous publications (Nunes et al., 2020), we did observe the

correlation that the reviewer asks. We were able to show that correct centrosome separation and positioning is essential to ensure both the timing of mitosis and the efficiency of chromosome segregation. We propose to clarify the text to reflect this information.

"It's also not clear why the authors expected confinement to rescue the spindle defects imposed by STLC treatment (supp figure 5). An alternative hypothesis that the authors neglect to mention is that faster cyclinB1 entry into the nucleus could also induce defects through changes to nuclear events such as chromosome condensation? Did they also see any changes to the rate of chromosome condensation in the confined prophase? Either way, the authors should explain more clearly in the text what they think is happening here".

We agree that the data regarding treatment with STLC is not relevant to the message of the paper. We propose to remove this data from our manuscript. We did not observe significant changes in chromosome condensation in our experiments. We propose to clarify the text.

"No reference is cited for the endogenous tagged CyclinB1 RPE1 line nor are any details about its construction given. Has this cell line been previously published by the Pines lab? Are one or both alleles tagged? N or C terminus"?

We apologize for this oversight. This cell line has been previously used and published by the Pines lab (Collin et al., 2013) and other labs (Afonso et al., 2019). The original publication (Collin et al., 2013) has all the details regarding the tagging of cyclin B1 and generation of the cell line. We will clarify this information in the text.

"Figure 1C: presumably n in this case is number of experiments, not cells. How many cells were analysed in each case"?

As stated above for reviewer 1, we will clarify the number of cells/experiments in all cases.

"Figure 1H. Why do the graphs have different scales on the x axis? Where does 101+-12s for confined cyclin B translocation mentioned in the text come from? From the graph, it looks longer than this"?

We imaged cells in late G2 until they enter NEP. Because confinement triggers rapid mitotic entry, the x axes for confined cells is shorter than in controls. A direct comparison between unconfined and confined cells can be observed in fig. 1g. Note that the green lines corresponding to confined cells are shorted, as these cells enter mitosis prematurely.

The values for cyclin B1 translocation were obtained from datasets where the lowest nuclear cyclin B1 levels were normalized to 1 and not from fig. 1g. These data were fitted with an exponential function, allowing us to obtain the entry rate and half-time. We propose to clarify this information in the text.

"Figure 3 J, K. Confinement is able to rescue the effect of Y27 on cyclin B dynamics but not shROCK1. Why this difference? The authors should discuss this discrepancy in the text".
We apologize to the reviewer that we were not clear but there is no discrepancy in the data. We have treated cells with Y27632 with or without confinement (Fig. 3c, d, j). To confirm that interfering with ROCK does induce a defect in cyclin B1 translocation, we decided to deplete ROCK1 with shRNA (Fig. 3k). As expected, treatment with Y27632 or shROCK1 yields the same phenotype. However, we did not confine the cells treated with shROCK1. The reviewer was probably misled by our choice of color in the graphs. We will correct this.

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

"Increase in nuclear membrane tension has been shown to trigger cPLA2 recruitment to the NE (Enyeidi et al, 2013; Lomakin et al. 2020), although the authors show here that confinement does not induce cPLA2 recruitment (but still increases NE tension figure 4G) in the absence of Rock activity or when the LINC complex is disrupted. This is surprising considering that confinement should increase NE tension independently of actomyosin contractility and should increase cPLA2 recruitment at the NE, unless in this case cPLA2 recruitment is not mediated by an increase in NE tension".
The point raised by the reviewer is important. Indeed, previous papers (Enyeidi et al, 2013; Lomakin et al. 2020), clearly show an increase in cPLA2 recruitment following nuclear tension. However, it should be noted that those papers were based on exogenous expression of fluorescently-tagged cPLA2. On the other hand, our results were obtained by immunofluorescence analysis of the endogenous protein, which normally decreases the overall signal-to-noise ratio. Nevertheless, this comment from the reviewer prompted us to carefully reanalyze the data related to cPLA2 recruitment to the NE. As our data clearly shows, one of the most striking events that occurs during this G2-M transition is nuclear unfolding (fig. 4d-g), with a corresponding increase in nuclear area. We reasoned this increase in nuclear area could dilute the density of cPLA2 molecules on the NE (even if it was being recruited at higher levels), therefore reducing its fluorescence signal intensity. Such an effect was previously described for other proteins that are recruited to the NE, such as dynein (Boudreau et al., 2019; Nunes et al., bioRxiv preprint 2019).
To circumvent this limitation, we proceeded to measure integrated fluorescence intensity of cPLA2 on the NE and nucleoplasm, as this parameter takes into account both the total fluorescence intensity within a ROI, as well as area variations. This data has been added to figure 4e and h, replacing the original panels. As can be seen, with these new metrics it is possible to observe an increase in cPLA2 integrated fluorescence intensity on the NE following confinement, even after treatment with ROCK inhibitors or expression of DN-KASH. The text has been updated to reflect these changes.

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

"The specific contribution of Nuclear Envelope tension. The authors conclude that confinement acts through increasing NE tension, although confinement may affect cytoplasmic signaling, which could contribute to G2/M transition. The authors should test whether compressing the nucleus versus compressing the cytoplasm have distinct effects on cyclin B1 nuclear translocation and G2/M, as it has been done by others when addressing nuclear mechanosensitive mechanisms (Elosegui-Artola et al. or Lomakin et al.)".

We thank the reviewer for this comment. The experimental system we describe in our manuscript has been extensively used before to confine cells with high temporal resolution (Lancaster et al., 2013; LeBerre et al., 2014; Liu et al., 2015; Venturini et al., 2020). However, unlike the papers highlighted by the reviewer (Elosegui-Artola et al. or Lomakin et al.), which use an AFM setup to confine cells, our system lacks the spatial resolution to allow the compression of specific subcellular regions. This makes it impossible to test specific cytoplasmic compression under our experimental conditions. Moreover, the AFM technique that allows compression of subcellular structures requires very specialized equipment and training, which can only be performed in a restricted number of labs around the world. This makes it impossible to perform the experiments within the timeframe of a revision.

Nevertheless, we would like to highlight that our experimental data also strongly argues for a cytoplasmic signal in this process, namely from the cytoskeleton. This signal is particularly evident when cells are entering mitosis in unconfined situations. Accordingly, we show that seeding cells in stiffer substrates, widely accepted to increase cytoskeletal tension (Zhou et al., 2017; Doss et al., 2020; Mih et al., 2012, Trichet et al., 2011), affects cyclin B1 translocation (fig. 1i, j). Moreover, when we disrupt the LINC complex or interfere with actin and myosin, cyclin B1 translocation is also severely affected (fig. 3). All these observations point towards a significant contribution of the cytoskeleton to this process. Importantly, our data also goes in line with models proposed by others, showing that an increased tension on the NE leads to cPLA2 recruitment (Lomakin et al., 2020; Venturini et al, 2020), a process which seems to be independent of cytoplasmic kinase signaling (Enyedi et al., 2016). Notably, this increased NE tension can modify nuclear pore opening (Zimmerli et al., 2021), possibly facilitating nucleocytoplasmic shuttling of transcription factors (Aureille et al., 2019; Elosegui-Artola et al., 2017). Based on our data and these previous reports, we believe a similar mechanism allows the fine-tuning of cyclin B1 nuclear translocation, therefore regulating mitotic entry.

June 17, 2022

Re: JCB manuscript #202205051T

Dr. Jorge Ferreira
Instituto de Investigação e Inovação em Saúde - i3S
Rua Alfredo Allen
Porto 4200-135
Portugal

Dear Dr. Ferreira,

Thank you for submitting your manuscript entitled "Mechanosensitive control of mitotic entry". Your manuscript has been assessed by one additional reviewer recruited by JCB and their comments are appended below. We thank you for your patience during the review process. Although the reviewer expresses potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you will see, the main concern of this reviewer is that the 'time zero' in your experiments is defined in the absence of a quantitative molecular measure of the cellular state, which may affect the results and their interpretation. We agree with this reviewer with regard to the need of accurately defining the parameters used to set 'time zero' in your experiments, for example using endogenously tagged cyclin B1 RPE-1 cells to distinguish between early and late G2 cells, as suggested in their review. We understand that repeating some key experiments in a new cell line entails a substantial amount of time and effort, but, in our view, this is a critical point and thus we would like to emphasize its importance. This reviewer also asks for clarification on which molecular event is used to define entry into mitosis and for additional controls to exclude that the nuclear barrier is not leaking under confinement. Thus, in addition to your proposed revision plan to address the concerns of the reviewers nominated by Review Commons, addressing all the points raised by the JCB reviewer, first and foremost the 'time zero' point of concern, will be required.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. The typical timeframe for revisions is three to four months, but we are flexible if more time is needed -please, contact us to request an extension. 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.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count 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.

Figures: Your manuscript may have up to 10 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.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Your manuscript 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.

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. 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 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.

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

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Ulrike Kutay
Monitoring Editor
Journal of Cell Biology

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

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

The manuscript entitled "Mechanosensitive control of mitotic entry" from Dantas et al. explores a contribution of mechanical forces to promote commitment to mitosis.
Notably, the authors propose that actin contractibility acts on the nuclear envelope at the G2/M transition, stimulating cyclin B1 nuclear import and timely mitosis onset.

Overall, I found this work interesting, but I have several main concerns about the data and control conditions, as detailed below. Also, I found the manuscript globally under-referenced about previous works on cyclin B1 nuclear import (some references are listed below).

Main points:

I. My first concern is the following: the authors through a set of experiments defined "time zero" to analyze cyclin B1 nuclear translocation and so mitotic entry as the time of lowest cyclin B1 fluorescent signal in the nucleus. Then , using several inhibitory compounds (see for instance Fig. 3i to 3m), the authors claimed that cyclin B1 nuclear translocation was inhibited by the treatment.
How can authors distinguish between a cell in late G2 in which cyclin B1 translocation is inhibited by the treatment from a cell in early G2 in which cyclin B1 translocation will not take place ?, using this "time zero" parameter.
Distinction between early and late G2 cells using endogenously tagged cyclin B1 RPE-1 cells (the present work) as been previously described by Feringa et al. Nat. com. 2016.
DOI: 10.1038/ncomms12618.
Also, it was unclear which event is reproducibly taken into account to define entry or not into mitosis in the different experiments: chromosome condensation, cyclin B1 import, NEBD, or spindle formation ?

II. To exclude that the nuclear barrier is not leaking under confinement, a key point for the conclusion of the present work, additional control experiments should be performed, such as using a soluble nucleoplasmic NLS-GFP construct and quantifying the cytoplasmic fluorescence signal before and after confinement.

III. The variability in the kinetics of cyclin B1 import between experiments on the same cells is puzzling: from 700 sec. in Fig. 1g,

h; 300 sec. in Fig. 2; 150 sec in sup. Fig. 2.
Could the authors comment on that?
Also, is the equilibrium (ratio) between nuclear and cytoplasmic cyclin B1 reached in late prophase modified under confinement?, as suggested on Fig. 1g, indicating that confinement do not act only on the rate of nuclear translocation.

IV. A previous work from Pines's lab showed that active cyclin B1 - Cdk1 triggers its import at prophase onset. DOI: 10.1083/jcb.200909144. Can the authors speculate on how confinement forces cyclin B1 translocation upon Cdk1 inhibition (RO-3306).

Others points:

. On Fig.2h, a delay in cyclin B1 nuclear import upon Leptomycin B treatment is not visible as claimed by the authors.

. Fig.3 j; l and m: Control experiments (DMSO) are missing.
Similarly, to conclude that AAOCF3 decreases cyclin B1 nuclear translocation (Fig. 4i), control experiment must be provided.

. Sup Fig. 3 a: statistical analysis is missing
. Sup Fig. 3b: Can the authors explain how is defined t0 ?
. Sup Fig. 3c: (See also point I). Are cyclin B1 5A-GFP expressing cells in early or late G2 ?

Minor:

Materials and methods section:
I will help the reader to clarify for the different cell lines used whether they express endogenous or exogenous fluorescent-tagged proteins.

References are missing for SMARTpool siRNA, Addgene tubulin-mRFP construct, all antibodies used.

Cell lines: "30 µg of Lipofectamin 2000" Is it instead 30 µl ?

Point-by-point response to reviewers - manuscript #202205051T

Reviewer 1 (Review Commons)

"The specific contribution of Nuclear Envelope tension. The authors conclude that confinement acts through increasing NE tension, although confinement may affect cytoplasmic signaling, which could contribute to G2/M transition. The authors should test whether compressing the nucleus versus compressing the cytoplasm have distinct effects on cyclin B1 nuclear translocation and G2/M, as it has been done by others when addressing nuclear mechanosensitive mechanisms (Elosegui-*Artola et al. or Lomakin et al.)".*

We thank the reviewer for this comment. The experimental system we describe in our manuscript has been extensively used before to confine cells with high temporal resolution (Lancaster et al., 2013; LeBerre et al., 2014; Liu et al., 2015; Venturini et al., 2020). However, unlike the papers highlighted by the reviewer (Elosegui-Artola et al., 2017 or Lomakin et al., 2020), which use an AFM setup to confine cells, our system lacks the spatial resolution to allow the compression of specific subcellular regions. This makes it impossible to specifically test cytoplasmic compression under our experimental conditions. Moreover, the AFM technique that allows compression of subcellular structures requires highly specialized equipment and training, which can only be performed in a very restricted number of labs around the world (namely, the ones mentioned by the reviewer). Moreover, although both papers mentioned by the reviewer (Elosegui-Artola et al., 2017 or Lomakin et al., 2020) do perform cytoplasmic compression, they are completely different in their experimental setup, which precludes any direct comparison between them. Overall, these difficulties make it impossible to perform the experiments within the timeframe of a revision.

Nevertheless, we would like to highlight that our experimental data also strongly argues for a cytoplasmic signal in this process, namely from the cytoskeleton. This signal is particularly evident when cells are entering mitosis in unconfined situations. Accordingly, we show that seeding cells in stiffer substrates, widely accepted to increase cytoskeletal tension (Zhou et al., 2017; Doss et al., 2020; Mih et al., 2012, Trichet et al., 2011), affects cyclin B1 translocation (new Sup. Fig. fig. 2A, B). Moreover, when we disrupt the LINC complex or interfere with actin and myosin, cyclin B1 translocation is also severely affected (fig. 3). All these observations point towards a significant contribution of the cytoskeleton to this process. Importantly, our data also goes in line with models proposed by others, showing that an increased tension on the NE leads to cPLA2 recruitment (Lomakin et al., 2020; Venturini et al, 2020), a process which seems to be independent of cytoplasmic kinase signaling (Enyedi et al., 2016). Notably, this increased NE tension was recently shown to modify nuclear pore opening (Zimmerli et al., 2021), possibly facilitating nucleocytoplasmic shuttling of transcription factors (Aureille et al., 2019; Elosegui-Artola et al., 2017; Andreu et al., 2022). Based on our data and these reports, we believe a similar mechanism allows the fine-tuning of cyclin B1 nuclear translocation, therefore regulating mitotic entry.

"To consolidate their model, the authors should also test whether decreasing NE tension (independently of actomyosin tension) has opposite effect on G2/M (for example using LBR *overexpression)".*

We have overexpressed LBR-RFP in our RPE-1 cyclin B1-Venus cell line and measured cyclin B1 nuclear translocation, in comparison to unmanipulated cells. These results have been added to the manuscript (Fig 5). As can be observed from the data, overexpression of LBR significantly impairs cyclin B1 nuclear translocation rate. This indicates that while increased nuclear tension can accelerate cyclin B1 translocation, decreasing nuclear stiffness seems to have the opposite effect. In fact, LBR overexpression not only affected cyclin B1 translocation, but also decreased cPLA2 recruitment to the NE, which we have also added to Fig. 4I, J. This observation is in agreement with a previous report showing that LBR overexpression decreased ARA production (Lomakin et al., 2020) and therefore likely reflects cPLA2 inactivation.

*"Increase in nuclear membrane t*ension has been shown to trigger cPLA2 recruitment to the NE (Enyeidi et al, 2013; Lomakin et al. 2020), although the authors show here that confinement does not induce cPLA2 recruitment (but still increases NE tension figure 4G) in the absence of Rock activity or when the LINC complex is disrupted. This is surprising considering that confinement should increase NE tension independently of actomyosin contractility and should increase cPLA2 recruitment at the NE, unless in this case cPLA2 recruitment is not *mediated by an increase in NE tension".*

The point raised by the reviewer is important. Indeed, previous papers (Enyeidi et al, 2013; Lomakin et al. 2020), clearly show an increase in cPLA2 recruitment following nuclear tension. However, it should be noted that those papers were based on exogenous expression of fluorescently-tagged cPLA2. On the other hand, our results were obtained by immunofluorescence analysis of the endogenous protein, which normally decreases the overall signal-to-noise ratio. Nevertheless, this comment from the reviewer prompted us to carefully reanalyse the data related to cPLA2 recruitment to the NE. As our data clearly shows, one of the most striking events that occurs during this G2-M transition is nuclear unfolding (fig. 4A, B and Sup. Fig. 1H, I), which increases nuclear area. We reasoned this increase in nuclear area could dilute the density of cPLA2 molecules on the NE (even if it was being recruited at higher levels), therefore apparently reducing its fluorescence signal intensity. Such an effect was previously described for other NE proteins during prophase, such as dynein (Boudreau et al., 2019 and our unpublished observations). To circumvent this possible limitation, we proceeded to measure integrated fluorescence intensity of cPLA2 on the NE and nucleoplasm, as this parameter takes into account both the total fluorescence intensity within a ROI, as well as its area. This data has been added to figures 4E, J and M, replacing the original panels. As can be seen, using integrated fluorescence intensity measurements we can observe an increase in cPLA2 on the NE following confinement, even after treatment with ROCK inhibitors or expression of DN-KASH. The text has been updated to reflect these changes.

*"NPC transport ve*rsus NE permeability. The authors suggest that confinement increases cyclin B1 transport via NPC-mediated transport and rule out that confinement may affect NE permeability based on the absence of NE rupture using the INM marker lap2. However, the sample size for this observation is missing and NE permeability could be altered even in the

absence of major INM rupture observed by confocal. The authors should use a reporter of nuclear permeability (fluorescent cytoplasmic marker or nuclear marker as previously used by Denais et al or, 2016 or Raab et al., 2016) to make sure that NE permeability is not affected by confinement. In addition, NPC function should be tested in parallel with other fluorescent reporter (such as NLS-GFP constructs) to test whether global NPC-mediated transport is *changed during prophase (with or without confinement)".*

Again, an important point raised by the reviewer that relates to the integrity of the nuclear barrier. To address this, we expressed GFP-cGAS in RPE-1 cells with or without confinement and measured its association with DNA. Importantly, we confirmed that an 8μm confinement does not trigger cGAS association with DNA. This was further confirmed by analysing RPE-1 cells expressing NLS-GFP and tubulin-RFP, with or without confinement. Again, under an 8μm confinement, we did not observe leakage of NLS-GFP to the cytoplasm. These data were added to Fig S1. On the contrary, a 3μm confinement, was sufficient to induce leakage of NLS-GFP, as well as cGAS association with DNA. Overall, this allows us to conclude that our confinement setup increases cyclin B1 translocation but does not induce nuclear envelope rupture. We also increased sample size for our RPE-1 cells expressing Lap2β-RFP, but now use this group only as a read-out for confinement-induced nuclear unfolding. These data were also added to Fig. S1 and are discussed in the text.

"Effect of confinement on cyclin B transport (NEP) in adherent cells. In figure 1D, we can see that confinement enhances cyclin B1 nuclear translocation in cells adhering on fibronectin. Although it is unclear whether confinement has a significant effect in other figures, for example in figure 2F: DMSO is not significantly different from confiner+CDKi (same thing in 3i and 3j with Rock inhibitor and Kash construct). In these figures the untreated+confiner (or control in 3j) is missing, and the absence of difference between treated+confiner and control is puzzling*"*. Either there is no difference between confiner and CDKi+confiner and it means there is no difference between control and confiner (surprising considering figure 1D); or there is a difference between CDKi+confiner and confiner, indicating that CDK inhibition affects confinement-induced cyclin B import. Both possibilities suggest that the authors should significantly revisit their model*"*.

We thank the reviewer for this comment and understand the points raised. We have now added controls (DMSO) as well as DMSO+confiner to all graphs. As can be seen, the DMSO+confiner groups are always significantly different from the treatment+confiner groups. We believe this gives us a good readout of the specific contribution of individual targets to the process of confinement-induced cyclin B1 translocation. As an example, treatment with importazole, which blocks importin β activity, completely impairs cyclin B1 translocation (Fig. 2G) and is not rescued by mechanical confinement. This indicates that importin β is essential for the mechanical stimulation of cyclin B1 translocation. On the other hand, it is known that CDK1 inhibition alone abolishes cyclin B1 transport due to lack of CDK1 activity (Gavet and Pines, 2010). In addition, active cyclin B1-CDK1 can stimulate its own nuclear import, promoting a faster translocation (Gavet and Pines, 2010; Lindqvist, 2010). Therefore, it is possible that confinement forces the entry of some active cyclin B1 molecules through the NPCs, which then triggers a positive feedback mechanism, allowing more cyclin B entry. This partially restores cyclin B1 translocation to values similar to DMSO (Fig. 2F), however it is not sufficient to induce NEP (Fig. 3). We believe these differences between DMSO+confiner and CDK1i+confiner highlight the specific contribution of

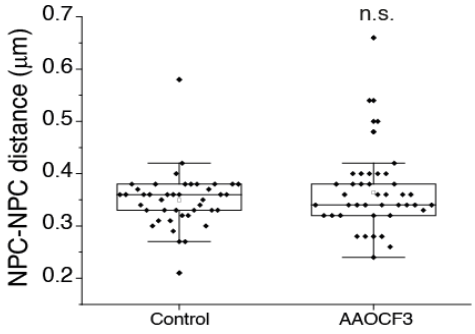
CDK1 activity to the process of confinement-induced translocation. We have now updated the text and discussed these points in the manuscript.

*"*In any case, all control (untreated, treated +/- confiner should be in all figures to avoid any *misunderstanding)".*

We have now added DMSO and DMSO+confiner groups to Figures 2 and 3.

"Consequences of cPLA2 recruitment at the NE. The authors state that "Active cPLA2 then stimulates actomyosin contractility creating a positive feedback loop" But the NE is already unfolded and distance between NPR is increased before cPLA2 recruitment. Does PLA2 inhibition affect nuclear irregularity (or distance between NPC)? Or does cPLA2 impact cyclin B1 transport via a distinct mechanism? Did the author analyze CDK1 phosphorylation in *presence of PLA2 inhibitor"?*

The sentence "active cPLA2 then stimulates actomyosin contractility creating a positive feedback loop" was written taking into consideration previous publications (Venturini et al., 2020; Lomakin et al., 2020), which demonstrate that confinement triggers cPLA2 NE recruitment and subsequent increase in actomyosin contractility. Our data shows that nuclear unfolding, NPC distance increase and cPLA2 recruitment occur approximately within the same timeframe. However, we did not perform experiments to accurately determine the spatiotemporal organization of these events. Therefore, to better characterize our system, we have now performed measurements of nuclear irregularity and NPC-NPC distance after cPLA2 inhibition with or without confinement. We have added this data to Fig. 4. As can be seen, cPLA2 inhibition increases nuclear irregularity, albeit not to the same extent as actomyosin inhibition (Fig. 4H). cPLA2 inhibition also prevents its recruitment to the NE. Overall, these observations suggest that active cPLA2 is required to trigger the actomyosin-dependent nuclear unfolding. However, inhibition of cPLA2 does not significantly decrease NPC distance, when compared to controls. Below we added a direct comparison of NPC distance between controls from Sup Fig. 1K and AAOCF3-treated cells.



According to the reviewer`s suggestion, we also investigated if cPLA2 might affect CDK1 phosphorylation. For that purpose, we analysed the pattern of CDK1 Y15 phosphorylation following cPLA2 inhibition. Phosphorylation of CDK1 on T14/Y15 is well known to prevent mitotic entry (Parker et al., 1992; Mueller et al., 1995; Kornbluth et al., 1994). We reasoned that if indeed cPLA2 affected the biochemical pathway

controlling CDK1 activation, we should see an increase in its inhibitory phosphorylation, following treatment with AAOCF3. As can be seen, treatment with AAOCF3 does increase the levels of CDK1 Y15. We have added this data to Sup. Fig. 3 and discussed it in the text.

"Robustness of the main observation. On page 4, the authors report that cells enter mitosis after 140 sec (+/- 80 sec) of confinement, although in the example showed in figure 1b, the cell enters at least 420 sec min after confinement, as we can see that the cell is already confined -420 sec (compressed shape) and NEP occurs at 0. Did the author showed a cell that was not included in their statistics? This would be very surprising considering the very low sample size used for this experiment (n=6 and 10). In addition, many observations have been made on small sample size (n=6 for figure 1) or/and not from independent experiments. The authors should increase their sample size and compare results from independent experiments *to consolidate their model"*

This experimental setup is very challenging, due to the fact that it is extremely difficult to find cells seeded in PEG that are in prophase and about to enter mitosis. This is probably due to the lack of adhesion, that significantly affects cell cycle progression (Huang et al., 1998; Benaud and Dickson, 2001; Lwin et al., 2007). Nevertheless, we agree with the reviewer that additional measurements were required. We have now increased sample size for cells seeded in PLL-g-PEG (n=23) and PLL-g-PEG +confinement (n=25) and added the data to the corresponding figures (Fig. 1A-C). As per the comment of reviewer 2, we have also added an experimental group with cells seeded in fibronectin (FBN; n=23), which readily enter mitosis. Based on these quantifications, we have determined that cells in PEG+confiner enter mitosis approximately within 260+/-129 sec. We also changed the representative image of cells seeded in PLL-g-PEG + confiner with one that is better fitting with the quantifications.

"2h shows nuclear signal (cyclin in grayscale), while 2e does not, why"?

We have grayscale images for cyclin B1 in all experimental conditions in new Fig. 2A-F.

"starting point to quantify cyclin entry is the lowest intensity, which may depend on many factors (and could be affected by experimental design). It would be necessary to have *synchronized cells to homogenize the starting point of these experiments".*

The majority of our cyclin B1 quantifications are normalized for the lowest value inside the nucleus. The reason for this is because prior to the increase in CDK1 activity in late G2, cyclin B1 nuclear levels are residual due to a predominance of nuclear export (Hagting et al., 1998; Toyoshima et al., 1998). Therefore, any increase in cyclin B1 nuclear import or decrease in its export should be readily observed as an increase in nuclear cyclin B1 levels. Moreover, we also clearly show that cyclin B1 translocation is accelerated upon confinement, even when we plot nuclear cyclin B1 intensity relative to NEP (new Figure 1J-L). We understand that synchronizing cells has been widely used in the past. However, we tend to avoid unnecessary manipulation of cells. In fact, protocols commonly used for cell synchronization in G2 involve either CDK1 inhibition (which is not recommended in this situation, as it directly affects the kinase controlling

cyclin B1 translocation) or a double thymidine block, that can cause DNA damage (Darzynkiewicz et al., 2011). Importantly, DNA damage is known to cause delays in cyclin B1 translocation (Toyoshima et al., 1998). Notwithstanding, we did not randomly choose the cells for our experiments. Our selection of cells was based on three well-defined parameters, previously shown to be reliable markers to identify cells in late G2/early prophase (Akopyan et al., 2014; Feringa et al., 2016). Namely, we quantified cells that (1) presented high cytoplasmic values of cyclin B1, (2) have no detectable levels of nuclear cyclin B1, and (3) the centrosomes had already separated. In combination, these allow the accurate identification of cells that are in late G2/early prophase, that we analysed in this study. We added quantifications of cytoplasmic cyclin B1 and centrosome distance to Fig. 1 that support our selection criteria and the text has been clarified accordingly. We hope these quantifications and clarifications satisfy the reviewer´s concerns.

"DN-KASH have been transiently transfected for single cell experiments, how does the authors unsure that cell observed are transfected? Does it have a fluorescent tag, if s*o which one"?*

We thank the reviewer for pointing this out and apologize for the lack of clarity when describing the experimental setup originally. For this experiment, we had to perform two parallel setups. We firstly measured cyclin B1 translocation in an RPE-1 cell line expressing cyclin B1-Venus that was transfected with DN-KASH-RFP. This allowed us to directly select cells that were expressing DN-KASH for quantification purposes. In a parallel experiment, we transfected DN-KASH-RFP into the RPE-1 cyclin B1-Venus/tubulin-RFP and performed the same experiment. In this last condition, identification of the KASH signal was very difficult due to the RFP signal coming from the tubulin tag. However, our measurements for cyclin B1 translocation rates were very similar in both conditions and significantly lower than controls, strongly suggesting that these cells were indeed expressing DN-KASH. We have now clarified this in the text and added a panel (Fig. S3), showing translocation rates in both conditions.

"requires contact with external stimuli" or "that mechanical confinement is sufficient to overcome the lack of external stimuli." (page 4): external stimuli is vague here and it could be *better to replace it with a more specific description."*

We agree with the reviewer that external stimuli is somewhat vague. We have now clarified this sentence in the text by replacing "external stimuli" with "cell adhesion".

"The authors do not comment the potential overlap of their findings with other reports involving the LINC complex (Booth et al., ELife) or CDK-mediated actin remodeling (Ramanathan et al., *NCB 2015) during prophase"*.

We have now discussed our data considering the report on the LINC complex cited by the reviewer (Booth et al., ELife). However, it should be noted that the CDK1-mediated actin remodelling proposed by Ramanathan and colleagues occurs later on in mitosis, during prometaphase, when the mitotic cortex is being assembled. In fact, the authors use nocodazole-arrested cells to test the involvement of CDK1 in mitotic cortex assembly. While that does not preclude a role for CDK1 in actin remodelling during prophase, there is little evidence available. In earlier time points, the role of CDK1 in

cytoskeletal remodelling seems more likely related to focal adhesion disassembly (Jones et al., 2018, JCB).

Reviewer 2 (Review Commons)

"This work contains an impressive amount of data including some technically challenging experiments. The conclusions are convincing and for the most part well supported by experimental evidence (for exceptions see below). Appropriate controls are presented and statistical analysis is adequate. The methods are mostly described well but some important details are omitted (see below). The methods and figure legends would benefit from expansion, particularly in describing how the images presented relate to quantification in graphs*"*.

We thank the reviewer for this comment. We have improved and clarified the methods section and figure legends to link the images with the quantifications presented. We hope this has improved the understanding of the figures.

"Figure 1A-C: here the authors show that non-adherent cells only enter mitosis when confined. There is some key information lacking here, including the experimental timeframe. How long were the cells plated on pll-peg before imaging and for how long were they imaged? In 1C, 80% of confined cells enter mitosis, which implies that cells were filmed for a relatively long time (given an average cell cycle length of 20-*24 hours)".*

Similarly to what we explained in the previous point, we have now clarified how this experiment was performed. Importantly, in fig. 1C, it does not mean that 80% of the entire population entered mitosis. Instead, 80% of the filmed cells entered mitosis following confinement. As we were filming cells with high spatial and temporal resolution, we had to resort to single cell imaging for every movie and for shorter periods of time. As a result, cells in PLL-g-PEG only were filmed for a maximum of 3 hours. Cells in the PLL-g-PEG+confiner experimental group were individually selected and confined. These entered mitosis shortly after (approximately 260 sec after confinement), as pointed out in the text.

"Unless of course cells were previously synchronised in G2 but the authors do not state that this is the case. In the legend it states that images were acquired every 20s. Imaging cells for 20+ hours every 20s with multiple zs is likely to have a very deleterious effect on cells and to disrupt mitotic entry itself. The authors need to explicitly explain the experimental set-up used to generate *the graphs in figure 1".*

As stated in response to the other reviewers, we did not synchronize cells in G2. We understand that synchronizing cells has been widely used in the past. However, we tend to avoid unnecessary manipulation of cells. In fact, protocols commonly used for cell synchronization in G2 involve either CDK1 inhibition (which is not recommended in this situation, as it directly affects the kinase controlling cyclin B1 translocation) or a double thymidine block, that can cause DNA damage (Darzynkiewicz et al., 2011). Importantly, DNA damage is known to cause delays in cyclin B1 translocation (Toyoshima et al., 1998). However, we based our selection of cells in late G2/early prophase on well-defined criteria. Namely, elevated cytoplasmic levels of cyclin B1, undetectable levels of nuclear cyclin B1 and the separation of centrosomes. In

combination, these allow the accurate identification of cells in late G2/early prophase, as previously defined (Feringa et al., 2016; Akopyan et al., 2014). We have added this data to Fig. 1. Moreover, given that centrosome separation should occur approximately 10-20 min before NEP in an unperturbed cell cycle (Matthews et al., 2012; Gavet and Pines, 2010), the imaging conditions did not induce phototoxicity and allowed us to image cells with high spatiotemporal resolution. We have also clarified the experimental setup used in figure 1.

*"In 1C, it would also b*e good to see the equivalent adherent control included in the graph (ie *% cells that enter mitosis on fibronectin in the same timeframe)".*

We have quantified the percentage of cells seeded on fibronectin that enter mitosis and added this data to figures 1B and C.

"The authors use the data in 1A-C to claim that 'the G2-M transition requires contact with external stimuli'. However they haven't shown this, only that non adherent cells don't enter mitosis. To show that the G2/M transition is affected, they need to look at the cell cycle phase of cells on PLL-*PEG and show that cells become arrested specifically in G2".*

We agree that our data does not show a specific G2 arrest, as we did not carefully look at cell cycle progression. Moreover, it has been shown by others that loss of cell adhesion can induce a G1 arrest (Huang et al., 1998; Huang and Ingber, 2002; Lwin et al., 2007). Rather, our data shows, in accordance with one of our previous publications (Nunes et al., 2020), that efficient mitotic entry requires a sufficient adhesion area. We have rewritten the text to reflect this and clarified that cells do not enter mitosis in conditions of low adhesion.

*"*Figure 5: The explanation of the conclusions here was hard to follow. It's not immediately clear why a faster prophase would lead to chromosome attachment delays in metaphase or segregation errors in anaphase since these events occur only after NEP. I think the authors' hypothesis is that a faster prophase results in less time for centrosome separation and that this is responsible for later spindle defects but this is not very clearly stated. If this is the case, then one might expect cells in which centrosome separation is delayed to also be the cells with lagging chromosomes. Did *the authors observe such a correlation"?*

We apologize for the lack of clarity. As the reviewer rightly points out, a faster prophase means that NEP occurs before centrosomes have the time to properly separate and assemble a spindle. This has been previously shown by us and others to increase chromosome missegregation rates (Nunes et al., 2020; Silkworth et al., 2012; Kaseda et al., 2012). In fact, in one of our previous publications (Nunes et al., 2020), we did observe a correlation between the extent of centrosome separation and the presence of lagging chromosomes. We were able to show that correct centrosome separation and positioning are essential to ensure both the timing of mitosis and the efficiency of chromosome segregation. We have now clarified this information in the manuscript and propose that premature cyclin B1 entry forces NEP before centrosomes are correctly positioned.

"It's also not clear why the authors expected confinement to rescue the spindle defects imposed by STLC treatment (supp figure 5). An alternative hypothesis that the authors neglect to mention is that faster cyclinB1 entry into the nucleus could also induce defects through changes to nuclear events such as chromosome condensation? Did they also see any changes to the rate of chromosome condensation in the confined prophase? Either way, the *authors should explain more clearly in the text what they think is happening here".*

We agree that the data regarding treatment with STLC, as well as DHC RNAi is not central to the message of the manuscript and might create unnecessary confusion. We have now decided to remove this data from the manuscript.

Although we cannot rule out that a faster cyclin B1 translocation might affect other nuclear processes, we performed confinement in cells which were in late G2/early prophase and therefore already had well-condensed chromosomes. Under these conditions, we did not observe significant changes in chromosome structure. We have now clarified the text to reflect this.

"No reference is cited for the endogenous tagged CyclinB1 RPE1 line nor are any details about its construction given. Has this cell line been previously published by the Pines lab? Are one or both al*leles tagged? N or C terminus"?*

We apologize for this oversight. This cell line has been previously used and published by the Pines lab (Collin et al., 2013) and other labs (Afonso et al., 2019). The original publication (Collin et al., 2013) has all the details regarding the tagging of cyclin B1 and generation of the cell line. We have added this information to the text.

"Figure 1C: presumably n in this case is number of experiments, not cells. How many cells were analysed in each case"?

As stated above in our comments to reviewer 1, we have clarified the number of cells/experiments in all cases.

*"Figure 1H. Why do the graphs have different scales on the x axis? Where does 101+-*12s for confined cyclin B translocation mentioned in the text come from? From the graph, it looks *longer than this"?*

We imaged cells in late G2, until they undergo NEP. Given that confinement triggers rapid mitotic entry, the xx axis for confined cells is always shorter than for controls. A direct comparison between unconfined and confined cells can be observed in new Fig. 1I-L. Note that in Fig. 1J, the green lines, corresponding to the confined cells are shorter, as these cells enter mitosis faster (and therefore quantifications stop). Nevertheless, we have now adjusted the scale in the xx axis of controls to match that of the confined cells. The values for cyclin B1 translocation were obtained from datasets where the lowest nuclear cyclin B1 levels were normalized to 1 and aligned to this value. These data were fitted with an exponential function, allowing us to obtain the entry rate and half-time for controls vs confined. We have added this information and the corresponding graph to the text (Fig. 1M).

"Figure 3 J, K. Confinement is able to rescue the effect of Y27 on cyclin B dynamics but not *shROCK1. Why this difference? The authors should discuss this discrepancy in the text".* **We apologize to the reviewer for the lack of clarity. However, we believe there is no discrepancy in the data. We have treated cells with Y27632 with or without confinement (new Fig. 3C, D, J). To confirm that interfering with ROCK activity does induce a defect in cyclin B1 translocation, we decided to deplete ROCK1 with shRNA, as an alternative (Fig. 3F, K). As expected, treatment with Y27632 or shROCK1 yields the same phenotype. However, we did not confine the cells treated with shROCK1. Therefore, we cannot directly compare confinement effects on Y27632- and shROCK1-treated cells. The reviewer was probably misled by our choice of colour in the graphs. We have updated line colours to help the analysis.**

Reviewer 1 (JCB)

I. My first concern is the following: the authors through a set of experiments defined "time zero" to analyze cyclin B1 nuclear translocation and so mitotic entry as the time of lowest cyclin B1 fluorescent signal in the nucleus. Then, using several inhibitory compounds (see for instance Fig. 3i to 3m), the authors claimed that cyclin B1 nuclear translocation was inhibited by the treatment. How can authors distinguish between a cell in late G2 in which cyclin B1 translocation is inhibited by the treatment from a cell in early G2 in which cyclin B1 translocation will not take place ?, using this "time zero" parameter. Distinction between early and late G2 cells using endogenously tagged cyclin B1 RPE-1 cells (the present work) as been previously described by Feringa et al. Nat. com. 2016. DOI: 10.1038/ncomms12618.

We thank the reviewer for this accurate comment and helpful suggestion and apologize for the lack of clarity in our manuscript regarding this point. In fact, for fig. 1, time zero corresponds to NEP and not the lowest nuclear fluorescence intensity. Nevertheless, we absolutely agree that if we based our analysis on a random selection of cells, there would be no way of distinguishing between cells in late G2/prophase from early G2 cells. For that reason, all cells were selected based on three parameters: (1) the elevated levels of cytoplasmic cyclin B1, (2) the absence of cyclin B1 in the nucleus and (3) the separation of centrosomes. These were used as proxy to determine whether the cells were in the correct cell cycle stage. We have now added the quantifications of cyclin B1 intensity and centrosome distance to Fig. 1, in support of our selection criteria. We have also clearly stated in the text how cells were selected and how quantifications were normalized.

Also, it was unclear which event is reproducibly taken into account to define entry or not into mitosis in the different experiments: chromosome condensation, cyclin B1 import, NEBD, or spindle formation ?

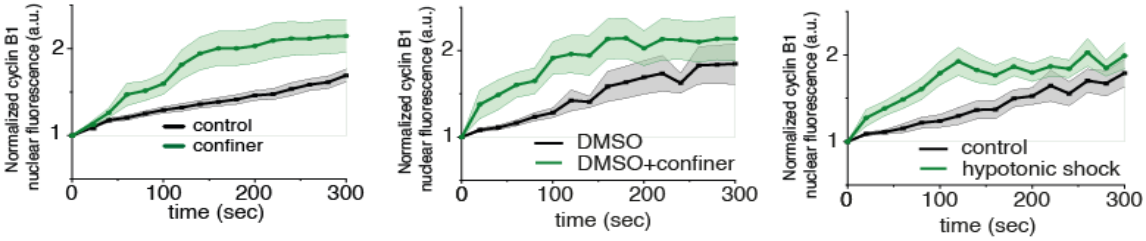
Irreversible entry into mitosis is usually defined as the moment when the nucleus loses its barrier function, allowing cytoplasmic proteins to invade the nuclear space. For this reason, we used the entry of soluble tubulin subunits into the nucleus as a proxy to determine NEP, in cells that are expressing tubulin-mRFP. This information is clearly stated in the text *"*We defined time zero as the lowest fluorescence intensity levels of nuclear cyclin B1 and quantified its increase as cyclin B1 translocated to the nucleus, up until the moment of tubulin entry. This last event marks the loss of nuclear barrier *function, which we defined as NEP".*

II. To exclude that the nuclear barrier is not leaking under confinement, a key point for the conclusion of the present work, additional control experiments should be performed, such as using a soluble nucleoplasmic NLS-GFP construct and quantifying the cytoplasmic fluorescence signal before and after confinement.

This is an important point also raised by another reviewer. To address this, we expressed GFP-cGAS in RPE-1 cells with or without confinement and measured its association with DNA. Importantly, we confirmed that an 8μm confinement does not trigger cGAS association with DNA. This was further confirmed by analysing RPE-1 cells expressing NLS-GFP and tubulin-RFP, with or without confinement. Again, under an 8μm confinement, we did not observe leakage of NLS-GFP to the cytoplasm. These data were added to Fig. S1. On the contrary, a 3μm confinement, was sufficient to induce leakage of NLS-GFP, as well as cGAS association with DNA. Overall, this allows us to conclude that our confinement setup increases cyclin B1 translocation but does not induce nuclear envelope rupture. We also increased sample size for our RPE-1 cells expressing Lap2β-RFP, but now use this group only as a read-out for confinement-induced nuclear unfolding. These data were also added to Fig. S1 and are discussed in the text.

III. The variability in the kinetics of cyclin B1 import between experiments on the same cells is puzzling: from 700 sec. in Fig. 1g, h; 300 sec. in Fig. 2; 150 sec in sup. Fig. 2. Could the authors comment on that?

Regarding the apparent differences highlighted by the reviewer, it should be noted that these graphs are not organized in the same way. Data in fig. 1g, h is normalized to the lowest value but aligned to the moment of NEP. This allows us to better visualize the correlation between cyclin B1 translocation and NEP, under confinement. On the other hand, the other graphs are aligned to the lowest value, allowing us to obtain a direct comparison of cyclin B1 translocation rates for all groups. The reviewer can find below the corresponding graphs for controls and different treatments, aligned to the lowest value that were mentioned by the reviewer.



As can be seen, they have similar patterns of variation, within equivalent time frames, indicating similar translocation rates. We have now added these graphs to Fig. 1 and Fig. S2 and clarified this information in the text.

Also, is the equilibrium (ratio) between nuclear and cytoplasmic cyclin B1 reached in late prophase modified under confinement?, as suggested on Fig. 1g, indicating that confinement do not act only on the rate of nuclear translocation.

To address this point, we have now quantified the ratio between nuclear and cytoplasmic (N/C) cyclin B1 immediately prior to NEP, with and without confinement. We added this analysis to Fig. 1N. As can be seen, confinement significantly increases the N/C ratio for cyclin B1. Interestingly, a recent report shows that cargo molecules with high molecular weight (>40kDa) undergoing facilitated transport have a higher mechanosensitivity for import than export, which results in an increased N/C ratio with force application (Andreu et al., 2022). Our cyclin B1-Venus has a high molecular weight (75kDa) and indeed exhibits higher N/C ratio upon mechanical stimulation, strongly suggesting a mechanosensitive response. However, at this point we cannot rule out that confinement acts on other aspects of the nucleocytoplasmic transport, such as the rate of export. Interestingly, the N/C ratio is no longer affected by confinement following treatment with leptomycin B, a known inhibitor of cyclin B1 nuclear export (Yang et al., 1998; Yang et al., 2001). This is indicative of a role for confinement also on the nuclear export rate. We have now added N/C quantifications to Fig. 1N and discussed this in the text.

IV. A previous work from Pines's lab showed that active cyclin B1 - Cdk1 triggers its import at prophase onset. DOI: 10.1083/jcb.200909144. Can the authors speculate on how confinement forces cyclin B1 translocation upon Cdk1 inhibition (RO-3306).

This observation from the reviewer is very interesting and relevant. Recently, it was shown that the nucleocytoplasmic transport of large proteins (>40 kDa) is a mechanosensitive process that favours import over export (Andreu et al., 2022). In support of this hypothesis, we now show that confinement increases cyclin B1 N/C ratio, compatible with a mechanosensitive process. While cyclin B1 translocation and mitotic entry still requires active CDK1 (as was previously shown by others and confirmed by us in this manuscript), we demonstrate that imposing a physical force on the prophase nucleus significantly accelerates cyclin B1 transport (Fig. 1). So how is cyclin B1 forced into the nucleus when CDK1 is inhibited? We believe, based on our data and previous reports, that imposing mechanical forces on the nucleus increases the diameter of nuclear pore complexes (Zimmerli et al., 2021; Elosegui-Artola et al., 2017). This opening of the nuclear pores, in combination with the mechanosensitive increase in nuclear import (Andreu et al., 2022) could allow the nuclear entry of cyclin B1 by increasing the permeability of the NPC barrier. We have now discussed these points in the manuscript.

On Fig.2h, a delay in cyclin B1 nuclear import upon Leptomycin B treatment is not visible as claimed by the authors.

On Fig. 2h, we can observe that the translocation for cells treated with Leptomycin B is very dampened, compared to controls or confiner. However, cyclin B1 translocation is not completely blocked. By aligning the quantifications to NEP, the translocation is clearly visible and occurs closer to the moment of mitotic entry. We have now added this information as a new graph to the manuscript (Fig. S3E) and rewritten the text to better reflect the effect on Leptomycin B on cyclin B1 nuclear transport.

Fig.3 j; l and m: Control experiments (DMSO) are missing. Similarly, to conclude that AAOCF3 decreases cyclin B1 nuclear translocation (Fig. 4i), control experiment must be provided.

We have now included DMSO controls to Fig. 3J, L and M. Similar DMSO controls have been added to Fig. 4.

Sup Fig. 3 a: statistical analysis is missing

A statistical analysis has been added to the graph. As can be seen, there is no significant difference between the two experimental groups.

Sup Fig. 3b: Can the authors explain how is defined t0?

We thank the reviewer for noting our oversight. Indeed, for the graph in Sup. Fig. 3b, t0 should have been defined as the moment of NEP. We have now updated the graph accordingly.

Sup Fig. 3c: (See also point I). Are cyclin B1 5A-GFP expressing cells in early or late G2 ? **As explained above, all cells were that were selected were expressing high levels of cyclin B1, undetectable nuclear levels of cyclin B1 and already showed separated centrosomes (including RPE-1 cyclin B1 5A-GFP). We have annotated the separated centrosomes in the corresponding figure (Sup Fig. 3c) to highlight this.**

Materials and methods section:

I will help the reader to clarify for the different cell lines used whether they express endogenous or exogenous fluorescent-tagged proteins.

We have now stated explicitly which cell lines are expressing endogenous or exogenous fluorescent-tagged proteins.

References are missing for SMARTpool siRNA, Addgene tubulin-mRFP construct, all antibodies used.

We have added references for these reagents.

Cell lines: "30 µg of Lipofectamin 2000" Is it instead 30 µl ?

We thank the reviewer for noticing this error. We have now corrected this sentence to 5µl of Lipofectamin 2000

August 24, 2022

RE: JCB Manuscript #202205051R

Dr. Jorge Ferreira
Instituto de Investigação e Inovação em Saúde - i3S
Rua Alfredo Allen
Porto 4200-135
Portugal

Dear Dr. Ferreira:

Thank you for submitting your revised manuscript entitled "Mechanosensitive control of mitotic entry". The reviewers have now assessed your revised manuscript and are overall satisfied with revisions. However, reviewer #1 still raises some important points that need text clarification and one minor experimental addition to Fig. S4. Thus, we would be happy to publish your paper in JCB pending final changes to address the remaining issues of reviewer #1 and 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. 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.

Please note that main text figures should be provided as individual, editable files.

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. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

4) Statistical analysis:
*** Error bars on graphic representations of numerical data must be clearly described in the figure legend.

The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).

Statistical methods should be explained in full in the materials and methods in a separate section.

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

*** Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a

separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). We are aware that you have provided this information in the 'Statistical analyses' section of Methods and in the figure legend itself, but this information seems to be missing in the corresponding legends of some of the figures (i.e. Fig. 1, Fig. 6, Fig. S1, Fig. S2 and Fig. S4)

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. Your current title, while fine, seems more like a review title, thus we would like to propose the following suggestion: "Nuclear tension controls mitotic entry by regulating cyclin B1 nuclear translocation"

6) Materials and methods:
Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. 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.
*** Please, indicate the imager (i.e. Lycor Odyssey) in which you have captured the signal of your western blot 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.

Please note that supplemental figures and tables 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 see any recent JCB paper for an example of this summary).

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."

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:
All animal and human studies must be conducted in compliance with relevant local guidelines, such as the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals or MRC guidelines, and must be approved by the authors' Institutional Review Board(s). A statement to this effect with the name of the approving IRB(s) must be included in the Materials and Methods section.

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

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

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

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

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

B. FINAL FILES:

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

-- 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,

Ulrike Kutay
Monitoring Editor
Journal of Cell Biology

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

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

Dantas and colleagues show that confinement stimulates G2/M transition in cells than can't adhere to their matrix. Investigating this further, the authors report that confinement and cell-generated contractility stimulate the nuclear translocation of cyclin B1. The authors made some important changes that improve their study and consolidate their results. However, I still have a few comments concerning the response to our initial review.
-Regarding the response to my initial comment on "contribution of Nuclear Envelope tension".
I realize that the authors do not have access to an AFM to perform the control experiment I suggested and test whether applying force specifically on the nucleus is sufficient to stimulate G2/M transition. Since confinement may trigger mechanosensitive cytoplasmic pathways which could contribute to G2/M transition (independently of the authors proposed pathway), it seems important to consider this limitation when the authors interpret the results obtained with the cell confiner. In addition, a few sentences remain vague in the revised result section and could be misleading. For example, the authors mention "nuclear stiffness" three times (changes highlighted in yellow, page 14 second paragraph and page 16), while nuclear tension seems more appropriate (LBR expression causes overproduction of NE membranes, leading to a decrease in NE tension). I would also suggest to precise some of the titles. This is a rapidly expanding field and I am worried that "mechanical stimulation" (title of the two first paragraphs in the result section) may be misleading, as it could describe many different types of mechanical stress/experimental systems. One possibility would be to replace it by a more precise wording indicating that it is confinement. One could also be misled by the main title and the use of "nuclear mechanics" in the abstract (nuclear mechanics is not probed nor investigated in this study).
-Regarding the response to my initial comment on "2h shows nuclear signal (cyclin in grayscale), while 2e does not, why"
The (same) grayscale panels were all already in the initial version of Figure 2 (there is no apparent difference between the initial and the revised version). I made this comment because the quantification in 2H shows that there is no nuclear signal in cells

treated with LMB, while the grayscale panel shows nuclear signal for cyclin B1. Can the authors explain why there is an apparent discrepancy between the figure (2E) and the quantification (2H)? It is also unclear why LMB has different consequences on cyclin B1 in figure 2H and in figure 1N. I understand that the quantification method is different between these two panels (nuclear versus nucleus/cytoplasmic ratio), although one would expect to see similar effect of LMB on cyclin B1 transport (and not opposite effect?) before NEP.
- Regarding the response to my initial comment on "Robustness of the main observation".
In the initial version, the authors showed in figure 1 an example of a cell entering mitosis at least 420 sec after confinement, while the revised statistics indicate that cells enter mitosis 260 +/- 129 sec after confinement. This indicates that the cell showed in the initial version was not included into the dataset, why was it excluded? In addition, I realize that these experiments are very challenging, but the revised statistics seem surprising (260 +/-129 sec) considering that the initial description stated 140 +/- 80 sec. Does the revised dataset include all previous results?
- Regarding the response to my initial comment on "DN-KASH have been transiently transfected for single cell experiments"
The authors should provide an image of RPE-1 cyclin B1-Venus cell line transfected with RFP-KASH (without fluorescent tubulin) as they performed this experiment for the corresponding analysis (the image provided in Supp. Fig 4 seems to be in regular RPE-1 cells, which were not used in the analysis). The authors used the same construct in Figure 4f, but it is unclear which channel overlap with RFP? Did the spectral overlap impact PLA2s analysis? There are no details on how this construct was made in the method section.
-Regarding the response to my initial comment on "Did the author analyze CDK1 phosphorylation in presence of PLA2 inhibitor"?
As the authors indicate, the panel in supplementary Fig. 4 suggests that cPLA2 inhibition affects CDK1 phosphorylation on Y15, although the total amount of CDK1 should be analyzed by western blot (on the same lysates) to allow proper interpretation (and to make sure that cPLA2 inhibition does not impact CDK1 expression).

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

The authors have fully addressed my comments. The revised manuscript is greatly improved in clarity and the additional experiments have strengthened the authors' conclusions. I highly recommend publication of this work in JCB.

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

In the revised manuscript, the authors answered the main points raised and provided sufficient data supporting their conclusion.

I still have few issues, as detailed below:

. In the first paragraph, the authors reported that cyclin B1 nuclear translocation occurs within 478 ({plus minus}102) sec in control conditions and is significantly accelerated under confinement. However, on the corresponding figure (Fig 1M) the time windows displayed is restricted to only 300 sec duration. The authors should provide a modified figure with the appropriate time scale.

. The authors mentioned in both introduction and discussion paragraphs that cyclin B1 expression increases as cells approach late G2/prophase, which is incorrect. According to previous data (see for example Feringa et al. 2016, Akopyan et al. 2014), cyclin B1 expression increases progressively during S and G2 phases.

Similarly, in both introduction and discussion paragraphs, it is mentioned that Wee1 is responsible for T14 phosphorylation of Cdk1, which relies on Myt1 but not Wee1 kinase. McGowan and Russell EMBO 1993; Liu et al. MCB 1997.

Dr. Ulrike Kutay

Monitoring Editor

Journal of Cell Biology

September 2nd, 2022

Re: JCB manuscript #202205051R

Dear Dr. Kutay,

I hope this letter finds you well. I would like to thank you for following our manuscript during this review process at the Journal of Cell Biology.

Please extend my warmest regards to the reviewers for their constructive input, which helped to greatly improve the manuscript. We have now concluded revising all the final points asked by the reviewers. You can find a point-by-point list of the changes included in the final version of the manuscript.

We hope this allows the publication of our paper in the Journal of Cell Biology.

With my very best regards,

Jorge G. Ferreira, PhD

Project Leader at Instituto de Investigação e Inovação em Saúde (i3S)
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Reviewer 1:

- The term “nuclear stiffness” was replaced with “nuclear tension”. We have also changed the title of the paper and some of the subsection headings to avoid confusing references to nuclear mechanics. We hope this clarifies the text.

- We have now specifically stated in the text that our confinement setup does not discriminate between compression of the cytoplasm and nucleus.

- Regarding the reviewer 1 doubts about leptomycin B (LMB), we apologize for our lack of a suitable explanation in the first review. I am afraid we did not completely understand the point raised by the reviewer. We would like to point out that the panel in Fig. 2H was used to determine translocation rates for cyclin B1. As was previously shown (Dos Santos et al., 2012), LMB treatment delays cyclin B1 nuclear accumulation. We believe this is reflected in the panel 2H, which shows a strong delay (but not complete block) in cyclin B1 accumulation. Indeed, at time=300sec, cyclin B1 levels are higher than at time=0sec (1.234 vs 1.000). However, since LMB treatment still allows cyclin B1 translocation, we can see cyclin B1 accumulate in the nucleus in the time points immediately prior to NEP (shown in Figs. 1N and 2E).

- Regarding the confinement of cells in PEG, we did not exclude any cells from our quantifications. We merely added more cells to our analysis (n=12), which increased the average time and the robustness of the observation.

- Regarding the DN-KASH experiment, the cell shown in Fig S4 is not of a parental RPE-1 cell, but an RPE-1 cell expressing cyclin B1-Venus/DN-KASH-RFP. We have now included the corresponding cyclin B1-Venus channel to the panel. For Fig. 4F, cPLA2 was conjugated with Alexa488 (green), DN-KASH-RFP (red) and LaminA/C conjugated with Alexa647 (infrared). Therefore, we did not observe any spectral overlap between the channels.

- We have performed WB detection using an anti-CDK1 antibody and added this to supplemental figure S4.

Reviewer 3:
- We have modified Fig. 1M to adjust the timings, as per the reviewer suggestion.

- We have corrected all the sentences highlighted by the reviewer both in the introduction and in the discussion.

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