An asymmetric junctional mechanoresponse coordinates mitotic rounding with epithelial integrity

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Re: JCB manuscript #202001042

Dr. Martijn Gloerich
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Utrecht 3584CG
Netherlands

Dear Dr. Gloerich,

Thank you for submitting your manuscript entitled "An asymmetric junctional mecanoresponse ensures epithelial integrity during mitotic rounding ". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that although the reviewers have voiced some enthusiasm for the study, they each raise a number of substantive concerns which preclude further consideration of the paper at this time. While the issues raised by reviewers appear to be addressable with further work, it seems unlikely that this can be completed in a typical revision period. Therefore, if you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

We would, however, be open to receiving an appeal which includes a significantly revised and extended manuscript that fully addresses each of the reviewers’ concerns in full. In particular, this would require providing further insight into the mechanisms by which mitotic cell rounding induces vinculin recruitment in neighboring cells, as voiced by reviewer #1 (a concern which was shared by the editors during initial evaluation). Such an extension of the study will likely require an expansion of the manuscript to a full Article.

If I may insert my own comments, the Borghi/Dunn tension sensor has the puzzling property that it registers tension in cadherins that are not engaged in cell-cell junctions. A construct analogous to the VE-cadherin sensor (module inserted at a different location) would be more likely to yield interpretable data. In any case, these experiments are less essential than developing further insight into mechanism.

Such a resubmission would be subject to evaluation for priority and novelty, as well as a second round of peer review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

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

In this manuscript Monster et al present data suggesting tension is present in the cell-cell junctions of mitotic epithelial cells. The tension is established when vinculin is recruited from neighboring cells to sites of mitotic cell contact with its neighbors. This is an interesting and well-written report that addresses an important area of cell biology that is not well understood. The data suggesting that tension is present in mitotic cell-cell junctions is well supported. However, there are several concerns that diminish enthusiasm for other aspects of the study.

There is significant concern with the use of MDCK cell lines which over-express GFP-vinculin. Multiple reports demonstrate that GFP-vinculin does not localize to cell-cell junctions when over-expressed in the presence of the endogenous protein. Consistent with this idea, the authors data indicate that tagged vinculin only localizes to cell-cell junctions when mitotic rounding is induced. Thus, the GFP-vinculin protein does not accurately recapitulate what is already known in the field, namely that vinculin localizes to adherens junctions in interphase cells. This concern is magnified by the fact that the studies showing that vinculin is recruited from neighboring cells to mitotic cell-cell junctions were done using co-cultures of the MDCK cells over-expressing GFP-vinculin in the presence of the endogenous protein and MDCK cells over-expressing mCherry-vinculin in the absence of the endogenous vinculin. Thus, it is possible that the authors observe mCherry-vinculin, but not GFP-vinculin, because the mCherry-vinculin integrates into junctions better. If recruitment of tagged vinculins is going to be assessed, then the tagged proteins should both be expressed in cells lacking endogenous vinculin.

Another weakness of the work is that manuscript is highly descriptive and no mechanistic insight into how mitotic cell rounding induces vinculin to be recruitment from neighboring cells.

The manuscript relies exclusively on microscopy-based techniques. The key assertions should be tested biochemically.

There is also concern that the work is not as rigorous as it could be. Specifically, GFP or mCherry controls are missing throughout. Controls for cell rounding induced specifically by mitosis are missing. Do cells induced to round by other mechanisms increase vinculin and myosin II localization to junctions? The authors indicate that vinculin recruitment to mitotic cell-cell junctions is specific as alpha catenin is not enriched. However, the images in Figure S1E indicate some tricellular accumulation of alpha catenin. Focal adhesions and cell-cell junctions are described but co-stains with markers of these adhesions are missing. The cell-cell junctions (and tight junction) markers are especially needed to assess the effects of alpha catenin mutant proteins on cell-cell adhesion in the interphase cells.
Reviewer #2 (Comments to the Authors (Required)):

This study seeks to address how epithelial integrity is maintained during cell division, where morphological rounding of the mitotic cell against its neighbors is shown to require a specialized asymmetric adhesive junction to accommodate both rounding of the mitotic cell and barrier function of the entire epithelium. This asymmetric adhesive junction requires the vinculin-binding region of α-catenin in cells adjacent to, but not within, the dividing cell. The authors build this model using MDCK cells lacking α-catenin and restored with mutants that block or enhance recruitment of vinculin to the adherens junction, together with a clever, heterologous cell mixing assay to interrogate the contribution of vinculin in dividing versus adjacent cells. Overall, the data are generally clearly presented and the model reasonably reflects the data. The manuscript is well written and scholarly, placing this work in context of the broader field. My comments to improve clarity of the message/model are indicated below.

Specific Points to address:

1. Fig. 1 asserts that cell-cell junctions are under increased tension during mitosis by inference, using the Geometric force inference (CellFit) analysis, which infers tension from the geometry of tri-/multi-cellular junctions. While I think this is reasonable, the field has tools to quantify tension on the cadherin catenin complex (CCC) directly using FRET sensor technology (Borghi... Dunn). Can the authors show changes in tension on the cadherin-catenin complex using this tool? Given the authors’ model that tension on the CCC appears asymmetric, can the authors do this using their clever, heterologous mixing assay? The prediction would be that the Borghi sensor FRET signal may be reduced at the surrounding cell interface. It would also allow the authors to interrogate if any changes are detectable in the dividing cell.

2. Minor: Fig. 1C legend should read Geometric forces INference (not interference)?

3. Related to Fig. 1: It is not clear to me that vinculin is truly recruited the mitotic cell/adjacent cell junction (i.e., from a cytosolic pool)-rather, that the entire apical junctional complex is pulled apically. Can the authors present the images in Fig. 1C in the xz direction, and at the same time drain the cytosolic GFP-vinculin stain with a permeabilization-before-fixation method? Similarly, if the authors use a membrane-localized GFP (as a control for GFP-vinculin), would they see the same recruitment? The small intestinal organoid image is quite nice/compelling (Fig. 1G), but given potential issues with antibody-epitope accessibility, it would be nice to see the same view in Fig. 1E.

4. The PDMS confinement experiment is quite elegant. Nonetheless, is it fair to say that the quantification in 2E would have to be true? Is the mitotic enrichment of GFP-vinculin due to the depth (z-direction) of the mitotic-cell/adjacent cell junction?

5. Fig. 3 is a really important experiment, as it may be of the first to link aCat binding to vinculin to TJ function under the perturbing effects of mitosis. Can this phenomenon be captured by TER measurements as well? I only ask because methods do not mention efforts to quantify events by blinding the observer/automation.

6. If possible, I would love to see the x-z images (like Fig 5F) of Figs 5A, 1E and 2C.

7. The vinculin mouse KO phenotype is known to be later than the aCat KO (i.e., preimplantation defect), and this difference has long be touted to undercut the potential importance of the vinculin/aCat interaction for tissue development. Given that a lot of epithelial cell divisions/morphogenesis take place between early embryo formation and late embryogenesis, could the authors comment on these data in light of their findings?
We thank the Reviewers for their evaluation of our work and their constructive criticisms, which helped us to improve the manuscript. We have performed additional experiments and revised the manuscript to address their criticisms in full, as detailed below in our point-to-point reply to their specific comments. We hope the Reviewers share our enthusiasm for our revised manuscript and find this appropriate for publication in The Journal of Cell Biology.

To better cover all the data presented in the revised manuscript, we have adjusted the title to “An asymmetric junctional mechanoresponse coordinates mitotic rounding with epithelial integrity” (instead of “An asymmetric mechanoresponse ensures epithelial integrity during mitotic rounding”).

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

In this manuscript Monster et al present data suggesting tension is present in the cell-cell junctions of mitotic epithelial cells. The tension is established when vinculin is recruited from neighboring cells to sites of mitotic cell contact with its neighbors. This is an interesting and well-written report that addresses an important area of cell biology that is not well understood. The data suggesting that tension is present in mitotic cell-cell junctions is well supported. However, there are several concerns that diminish enthusiasm for other aspects of the study.

There is significant concern with the use of MDCK cell lines which over-express GFP-vinculin. Multiple reports demonstrate that GFP-vinculin does not localize to cell-cell junctions when over-expressed in the presence of the endogenous protein. Consistent with this idea, the authors data indicate that tagged vinculin only localizes to cell-cell junctions when mitotic rounding is induced. Thus, the GFP-vinculin protein does not accurately recapitulate what is already known in the field, namely that vinculin localizes to adherens junctions in interphase cells. This concern is magnified by the fact that the studies showing that vinculin is recruited from neighboring cells to mitotic cell-cell junctions were done using co-cultures of the MDCK cells over-expressing GFP-vinculin in the presence of the endogenous protein and MDCK cells over-expressing mCherry-vinculin in the absence of the endogenous vinculin. Thus, it is possible that the authors observe mCherry-vinculin, but not GFP-vinculin, because the mCherry-vinculin integrates into junctions better. If recruitment of tagged vinculins is going to be assessed, then the tagged proteins should both be expressed in cells lacking endogenous vinculin.

Response:
We realize that in our original submission we had not been entirely clear in our description of the cell lines expressing exogenous Vinculin and our findings with these cells, for which we apologize.

In our cells expressing GFP-Vinculin on top of endogenous Vinculin, GFP-Vinculin is not completely absent from cell-cell junctions during interphase. Instead, we observe low levels of GFP-Vinculin at cell-cell contacts of interphase cells, with a very strong junctional enrichment of GFP-Vinculin during mitotic rounding, in particular at tricellular junctions (Fig. 2A and Supplementary Video 3). We would like to note that previous publications showed that the junctional levels of (endogenous) Vinculin in interphase can be influenced by the density of cells, with junctional Vinculin being higher in low density monolayers than at the cell density we have performed our experiments (e.g. Ibar et al., 2018; doi: 10.1242/jcs.214700). More importantly, we have directly addressed the Reviewer’s concern by monitoring GFP-Vinculin localization in the absence of endogenous Vinculin, by
expression of GFP-Vinculin in Vinculin knockout MDCK cells. While in these cells the localization of GFP-Vinculin at interphase cell-cell junctions was more clearly visible, importantly, the strong enrichment of GFP-Vinculin at cell-cell junctions following mitotic entry occurred in these cells as well (Fig. S2B-C and Supplementary Video 4). These results are supported by our immunostainings for endogenous Vinculin in MDCK cells, which show that although Vinculin is present at cell-cell contacts of interphase cells this is strongly increased in mitotic cells (Fig. 2G). Moreover, our data showing enrichment of Vinculin at mitotic cell-cell junctions are in line with mitotic cells being dependent on Vinculin recruitment to maintain an epithelial barrier, as MDCK monolayers that are unable to recruit Vinculin to cell-cell junctions predominantly show junctional leakage at mitotic cell-cell contacts (Fig. 4A-D). Thus, while we do not wish to negate the presence or role of Vinculin at interphase cell-cell junctions, our findings show that in MDCK epithelial cells the levels of junctional Vinculin increase as cells enter mitosis, which is important to maintain the epithelial barrier.

Regarding our mosaic experiments with differentially labeled Vinculin, we would like to clarify that the cells expressing mCherry-Vinculin did not lack endogenous Vinculin. As such, the GFP-Vinculin and mCherry-Vinculin cells in this experiment were thus comparable. Of note, the MDCK cells over-expressing mCherry-Vinculin in the absence of endogenous Vinculin were only used to test whether the effect of Vinculin knockdown on the integrity of mitotic cell-cell junctions could be rescued (Fig. S3E-G). We apologize for the lack of clarity, and have now described this more clearly in the text. Importantly, we observe a similar asymmetry in Vinculin recruitment at junctions between mitotic GFP-Vinculin cells and mCherry-Vinculin neighbors as with the reverse condition (with mitotic mCherry-Vinculin cells and GFP-Vinculin neighbors) (see below). To strengthen this point, we included these separated data in our revised manuscript (Fig. S4B). Moreover, we have included a control experiment with our mosaic cultures following treatment with HGF, which showed a symmetric recruitment of Vinculin from both of the neighboring cells forming the cell-cell junctions that is under elevated tension. This further supports that the observed asymmetry of Vinculin recruitment at mitotic junctions is not a consequence of differential ability of GFP- and mCherry-tagged Vinculin to be recruited to the cadherin complex (Fig. S4A).

Finally, the absence of the recruitment of Vinculin in the mitotic cell is in line with our findings in α-cateninΔVBS mosaic cultures, which revealed that the selective ability of Vinculin to bind α-catenin in neighboring cells is sufficient to maintain junctional integrity during mitosis (Fig. 5C-D).
We would like to note that to address the comments of Reviewer #2 to include xz-projections of our imaging data, in the revised manuscript we now use mScarlet-tagged Vinculin, as this fluorescent protein has superior yield, brightness and lifetime compared to mCherry (Bindels et al. 2017, doi: 10.1038/nmeth.4074). Since this did not change the outcome of any of our experiments, including the mosaic co-culture experiments (Fig. 5A-B, S4B and Supplementary Video 10), all our imaging experiments are now shown with mScarlet instead of mCherry-tagged Vinculin.

Another weakness of the work is that manuscript is highly descriptive and no mechanistic insight into how mitotic cell rounding induces vinculin to be recruitment from neighboring cells.

Response:
We have addressed this comment with a significant number of additional experiments to gain a more detailed understanding of the mechanism by which mitotic cell rounding induces Vinculin recruitment from neighboring cells.
Firstly, we performed extensive analyses of the morphology of epithelial cells, which shows that as cells round up during mitosis this induces local mechanical strain of the surrounding epithelium, with neighboring cells being pulled towards the mitotic cell (Fig. 1A, C-E). Secondly, we directly mapped tension within the epithelial layer using nanoscissor laser-cutting experiments. These experiments showed that as mitotic cells acquire their rounded morphology, they exert tensile forces on their neighbors (Fig. 1F-H, S1B and Supplementary Video 2). As we had shown previously, this corresponds to an increase in Vinculin localization at tricellular junctions formed by mitotic cells and their neighbors (Fig. 2A-B and Supplementary Video 3).

Thirdly, we added two key experiments that show that the tensile forces exerted by mitotic cells on their neighbors are directly responsible for the recruitment of Vinculin to mitotic cell-cell junctions. For this, we alleviated tension at cell-cell junctions linked to mitotic cells using our nanoscissor laser-cutting approach. This showed that upon relieve of tension GFP-Vinculin is released from junctions between mitotic cells and their neighbors (Fig. 2H-I and Supplementary Video 6). Moreover, to test the role of force transmission between mitotic cells and their neighbors further, we made use of MDCK cells in which endogenous cadherins are replaced with a truncated mutant (E-cadherin$^{151}$). E-cadherin$^{151}$ lacks a functional extracellular domain, but has an intact plasma membrane-tethered cytosolic tail that binds catenins and the actin cytoskeleton (Troxell et al., 2001; PMID: 10683147). Importantly, MDCK cells expressing this mutant retain a cohesive monolayer through the presence of other cell–cell junctions, but cannot transmit forces through E-cadherin adhesions between cells as a result of the truncated extracellular domain of E-cadherin$^{151}$. Essentially, we do not observe Vinculin recruitment to mitotic cell-cell junctions in E-cadherin$^{151}$ cells, indicating that E-cadherin-dependent force transmission between mitotic cells and their neighbors is required for this (Fig. S2F-G).
Finally, the above findings are in line with our previously described results, showing that the Vinculin-binding region within α-catenin that becomes exposed upon elevated tensile forces on cadherin adhesions is essential to localize Vinculin to mitotic junctions (Fig. 2F-G). Moreover, we excluded a contribution of two alternative mechanisms described for junctional Vinculin recruitment: i) we show that Vinculin enrichment is not a consequence of an increased presence of α-catenin (Fig. S2E), and ii) we show that Y822 phosphorylation of Vinculin that has been linked to junctional Vinculin regulation (Bays et al. 2014; doi:10.1083/jcb.201309092) is not involved in mitotic Vinculin enrichment, as a Vinculin Y822F mutant is still recruited to mitotic junctions (in 25/26 cells, both in the presence and absence of endogenous Vinculin, see below).

Altogether, our data demonstrate that rounding of mitotic cells exerts tensile forces on their neighbors, and that the force transmission through cadherin junctions between mitotic cells and their neighbors is responsible for Vinculin recruitment to α-catenin from the neighbor cells.

The manuscript relies exclusively on microscopy-based techniques. The key assertions should be tested biochemically.

Response:

We respectfully disagree with the Reviewer that our key assertions, i.e. the increased recruitment of Vinculin to E-cadherin in mitotic neighbors, can be validated biochemically. Importantly, the association between E-cadherin and Vinculin is dependent on tensile forces, and is therefore not well sustained following cellular lysis. Moreover, this tension-sensitive
binding occurs in the triton-insoluble fraction of the cells, which further hampers post-lysis analysis by co-immunoprecipitation. We would like to note extensive attempts by us and others at probing the spatiotemporal regulation of the E-cadherin/Vinculin interaction in cells with biochemical assays has to date been unsuccessful, for reasons described above. Equally important, the junctional enrichment of Vinculin investigated in our manuscript occurs locally, specifically at cell-cell junctions of mitotic cell neighbors. To the best of our knowledge these mitotic neighbors cannot selectively be isolated for biochemical analyses.

Therefore, our imaging-based techniques, which importantly are supplemented with mutational perturbations (E-cadherin\textsuperscript{T151}, α-catenin\textsuperscript{AVBS} and α-catenin\textsuperscript{CA}) and are now better controlled based on suggestions from this Reviewer (see below), are in our opinion uniquely suited to address the association of Vinculin with E-cadherin in mitotic neighbors.

There is also concern that the work is not as rigorous as it could be. Specifically, GFP or mCherry controls are missing throughout.

Response:
We have now included these controls, which show that unconjugated GFP is not enriched at mitotic cell-cell junctions (Figs. S2D and Supplementary Video 5). Moreover, through co-expression of GFP-Vinculin and unconjugated mScarlet in the same cells we show the complete absence of these control proteins at sites of Vinculin enrichment in mitotic cells (Fig. 2E). These control experiments demonstrate that the enrichment at mitotic junctions is specific to Vinculin.

Controls for cell rounding induced specifically by mitosis are missing. Do cells induced to round by other mechanisms increase vinculin and myosin II localization to junctions?

Response:
Although we agree that it would be interesting to test if the junctional enrichment of Vinculin due to cell rounding specifically occurs during mitosis, to the best of our knowledge there are no methods available to specifically induce rounding in (living) cells without disrupting cell-cell junctions themselves (as will for instance be the case with EGTA). We would like to note that during apoptosis cells also round up while they are being extruded from the epithelium. Interestingly, a recent publication showed a reduction in tension and of Vinculin at cell-cell junctions between apoptotic cells and their neighbors (Teo et al. 2020, doi: 10.1091/mbc.E20-01-0084). This may imply rounding-induced Vinculin enrichment could specifically occur at mitotic cell-cell junctions. However, because we cannot directly test the Vinculin response to other means of rounding, and the other example involves junctions between live and apoptotic cells, we would prefer not to include this statement on specificity in the manuscript.
The authors indicate that vinculin recruitment to mitotic cell-cell junctions is specific as alpha catenin is not enriched. However, the images in Figure S1E indicate some tricellular accumulation of alpha catenin.

**Response:** We have analyzed a large number of cells for α-catenin levels at the tricellular junctions of mitotic cells (Fig. S2E and Supplementary Video 1). This did not show any significant difference in the relative level of junctional α-catenin when cells enter mitosis. More specifically, we observed a random distribution of changes in α-catenin levels at the tricellular junctions following mitotic entry; while we did observe a minor increase in junctional α-catenin in a fraction of mitotic cells, a comparable fraction of cells showed a reduction of relative α-catenin levels instead. As such, we strongly believe that our data indicate that Vinculin enrichment is not a consequence of increased presence of α-catenin, but rather depends on its ability to bind Vinculin. We have now highlighted this further in the text describing these data (p7, line 1-4), and include an example image that is more representative of our data.

![S2E](image)

Focal adhesions and cell-cell junctions are described but co-stains with markers of these adhesions are missing. The cell-cell junctions (and tight junction) markers are especially needed to assess the effects of alpha catenin mutant proteins on cell-cell adhesion in the interphase cells.

**Response:** We have included these requested co-staining controls, which show that Vinculin in mitotic cells specifically localizes to adherens junctions visualized by E-cadherin immunostaining and not to focal adhesions visualized by Paxillin immunostaining (Fig. S2A). In addition, to further assess the junctional properties of α-cateninΔVBS cells during interphase, we have performed the requested immunostainings for the adherens junction protein β-catenin and tight junction protein ZO-1, which did not reveal any apparent differences in the organization of these cell-cell junctions between wildtype and α-cateninΔVBS cells in interphase (Fig. S3D). This is in line with our previously shown observation that selectively disrupting the recruitment of Vinculin to α-catenin does not induce strong effects on barrier integrity in interphase cells (Fig. 4C-D).
Reviewer #2 (Comments to the Authors (Required)):

This study seeks to address how epithelial integrity is maintained during cell division, where morphological rounding of the mitotic cell against its neighbors is shown to require a specialized asymmetric adhesive junction to accommodate both rounding of the mitotic cell and barrier function of the entire epithelium. This asymmetric adhesive junction requires the vinculin-binding region of α-catenin in cells adjacent to, but not within, the dividing cell. The authors build this model using MDCK cells lacking α-catenin and restored with mutants that block or enhance recruitment of vinculin to the adherens junction, together with a clever, heterologous cell mixing assay to interrogate the contribution of vinculin in dividing versus adjacent cells. Overall, the data are generally clearly presented and the model reasonably reflects the data. The manuscript is well written and scholarly, placing this work in context of the broader field. My comments to improve clarity of the message/model are indicated below.

Specific Points to address:

1. Fig. 1 asserts that cell-cell junctions are under increased tension during mitosis by inference, using the Geometric force inference (CellFit) analysis, which infers tension from the geometry of tri-/multi-cellular junctions. While I think this is reasonable, the field has tools to quantify tension on the cadherin catenin complex (CCC) directly using FRET sensor technology (Borghi... Dunn). Can the authors show changes in tension on the cadherin-catenin complex using this tool? Given the authors’ model that tension on the CCC appears asymmetric, can the authors do this using their clever, heterologous mixing assay? The prediction would be that the Borghi sensor FRET signal may be reduced at the surrounding cell interface. It would also allow the authors to interrogate if any changes are detectable in the dividing cell.

Response:
We agree with the Reviewer that in addition to our CellFit analysis it would be of great value to directly visualize tension on the cadherin complex using available FRET-based tension sensors. We have extensively tried this using both the E-cadherin- (Borghi et al. 2012, doi: 10.1073/pnas.1204390109) and α-catenin (Acharya et al. 2018, doi: 10.1016/j.devcel.2018.09.016) based TsMOD sensors, in collaboration with Dr. Nicholas...
Borghi, the author on the original E-cadherin TsMOD publication and with great experience in this approach. Unfortunately, for reasons unknown to us, we noted that as mitotic cells round up and change in height this greatly impacted our FRET measurements, and already strongly influenced FRET levels of tension-insensitive controls of our sensors. Unfortunately, we could therefore not convince ourselves that we could obtain reliable results with this method. We would like to note that although these junctional tension sensors have great potential, their success in measuring changes in tension at cell-cell junctions at single cell or even single junction resolution (to assess (a)symmetry in tension on cadherin junctions) thus far has been very limited.

Nonetheless, we acknowledge that it would be important to not merely infer junctional tension, but instead quantify this more directly. Therefore, we made use of two-photon laser nanoscissors to analyze junctional tension following mitotic rounding, determined by measuring junctional recoil following laser-ablation (Liang et al., 2016, doi: 10.21769/BioProtoc.2068). As predicted, tension significantly increased at cell-cell junctions that are connected to mitotic cells (Figs. 1F-H, S1B and Supplementary Video 2). This indicates that as cells undergo mitotic rounding they indeed exert tensile forces on their neighbors, resulting in Vinculin enrichment particularly at tricellular junctions between mitotic cells and their neighbors. Importantly, we experimentally demonstrate that this build-up of tension is essential for recruitment of Vinculin, as upon alleviation of tension at these neighboring junctions through nanoscissor laser ablation results in the release of GFP-Vinculin from mitotic cell-cell junctions (Fig. 2H-I and Supplementary Video 6).

Our current analysis of junctional tension does not allow us to address whether the distribution of tension is asymmetric at mitotic cell-cell junctions. In our opinion, investigating potential differences in tensile forces on the cadherin complex in mitotic cells would require a detailed proteomic, structural and/or super-resolution analyses of the cadherin complex within these mitotic cells. We would respectfully argue that this would be out of the scope of...
this manuscript, in particular since we are able to clearly demonstrate the importance of the asymmetry.

2. Minor: Fig. 1C legend should read Geometric forces INFERIENCE (not interference)?

Response:
We thank the Reviewer for noticing this mistake. Because we now directly analyzed junctional tension (see our answer to question 1), in our opinion the CellFit analysis that merely predicts junctional tension was not of any added value to our manuscript anymore. Given the limitations of this approach we have therefore omitted this from our revised manuscript.

3. Related to Fig. 1: It is not clear to me that vinculin is truly recruited the mitotic cell/adjacent cell junction (i.e., from a cytosolic pool)-rather, that the entire apical junctional complex is pulled apically. Can the authors present the images in Fig. 1C in the xz direction, and at the same time drain the cytosolic GFP-vinculin stain with a permeabilization-before-fixation method? Similarly, if the authors use a membrane-localized GFP (as a control for GFP-vinculin), would they see the same recruitment? The small intestinal organoid image is quite nice/compelling (Fig. 1G), but given potential issues with antibody-epitope accessibility, it would be nice to see the same view in Fig. 1E.

Response:
To address this question, we have redone all our live-cell imaging experiments using a confocal spinning disk microscope that allowed for imaging at high z-resolution. We have included the requested xz-projection in the manuscript, which validated that GFP-Vinculin becomes enriched at mitotic junctions, instead of a pre-existing pool of GFP-Vinculin being pulled more apically (Fig. 2C). We would like to note that all of our analyses on junctional Vinculin enrichment have now been performed on Z-projections of the entire cell-cell junction, ensuring none of our analyses could be affected by differences in the presence of junctional Vinculin within individual imaging planes. Moreover, the representative example images shown in the manuscript are also z-projections, which we have clarified in the text.

In addition, we have performed experiments in which we permeabilized the cells with CSK buffer prior to imaging to drain the cytosolic pool of GFP-Vinculin, in which the enrichment of GFP-Vinculin at mitotic junctions remained clearly present (see below). This indicates that the observed enrichment of Vinculin at mitotic cell-cell contacts truly represents an actin-associated junctional pool and not local accumulation of a cytosolic pool of GFP-Vinculin.
As a control for GFP-Vinculin being specifically enriched at mitotic cell-cell junctions, in line with the comment from Reviewer #1 we analyzed the levels of α-catenin in interphase and mitotic cells as a membrane-localized control. Importantly, this did not show any significant difference in the relative level of junctional α-catenin when cells enter mitosis (Fig. S2E and Supplementary Video 1). More specifically, we observed a random distribution of changes in α-catenin levels at the tricellular junctions following mitotic entry; while we did observe a minor increase in junctional α-catenin in a fraction of mitotic cells, a comparable fraction of cells showed a reduction of relative α-catenin levels instead. As such, we strongly believe that our data indicate that Vinculin enrichment is not a consequence of increased presence of α-catenin, but rather depends on its ability to bind Vinculin.

To exclude any potential issues with antibody-epitope accessibility in our small intestinal organoid immunostainings, we had performed co-immunostainings with E-cadherin (Fig. S5A). Moreover, the analysis of junctional Vinculin in these images is shown both as a ratio of junctional/cytosolic Vinculin (Fig. S5B), as well as the levels of junctional Vinculin staining relative to E-cadherin staining (Fig. S5C). We would like to note that because of the large amount of additional data that we included in our revised manuscript, we have now included the analyses of small intestinal organoids in the Supplementary Materials.

4. The PDMS confinement experiment is quite elegant. Nonetheless, is it fair to say that the quantification in 2E would have to be true? Is the mitotic enrichment of GFP-vinculin due to the depth (z-direction) of the mitotic-cell/adjacent cell junction?

Response:
The PMDS confinement experiments were already performed on a Spinning disc confocal microscope with high z-resolution. We have therefore now included xz-projection of this experiment, which confirmed that GFP-Vinculin becomes enriched at mitotic junctions and the observed increase in junctional signal is not due to changes in the depth of the mitotic junction and thus a pre-existing pool of Vinculin being observed only after PDMS release (Fig 3D). We have now also clarified in the text that the shown examples are z-projections and that we included the entire cell-cell junction along different imaging planes in our analyses.

Of note, in our revised manuscript we have included additional data showing that similar to our confinement experiments, preventing mitotic rounding by inhibition of actomyosin contractility prevents the enrichment of Vinculin at mitotic cell-cell junctions (Fig 3A-B and Supplementary Video 8).
5. Fig. 3 is a really important experiment, as it may be of the first to link α-catenin binding to vinculin to TJ function under the perturbing effects of mitosis. Can this phenomenon be captured by TER measurements as well? I only ask because methods do not mention efforts to quantify events by blinding the observer/automation.

**Response:**
Although this is an interesting implication, we would like to argue that our experiments do not show a direct role for α-catenin/Vinculin binding in TJ function. Instead, our experiments may indicate that adherens junctions need to be reinforced in order to retain intact cell-cell junctions between mitotic cells and their neighbors, and in the absence of strong adherens junctions this might indirectly influence the ability of TJ to be retained. TER-based analysis measures the barrier integrity of the entire monolayer and not individual cells. In our opinion it is therefore not feasible with TER measurements to determine loss of integrity in (the small number of) mitotic cells in the monolayer. As such, we think our imaging-based permeability assay is uniquely qualified to measure barrier integrity in the epithelium while making distinctions between mitotic and non-mitotic cells. Importantly, analysis of the streptavidin leakiness assay had been performed in an unbiased manner, similar to all other quantifications. We apologize for the lack of clarity on this and have now indicated this in the Methods.

6. If possible, I would love to see the x-z images (like Fig 5F) of Figs 5A, 1E and 2C.

**Response:**
As addressed in our answer to question #3, we have redone all of our live-cell imaging experiments using a confocal spinning disk microscope that allowed for high resolution analyses of cells in the z-direction, and we included xz-images for all of these Figures in our manuscript as suggested by the Reviewer (Fig. 2C, 3D, S4D).

7. The vinculin mouse KO phenotype is known to be later than the aCat KO (i.e., preimplantation defect), and this difference has long be touted to undercut the potential importance of the vinculin/aCat interaction for tissue development. Given that a lot of epithelial cell divisions/morphogenesis take place between early embryo formation and late embryogenesis, could the authors comment on these data in light of their findings?
Response:
We thank the Reviewer for raising this important question that we had not addressed in the initial submission. Given the fact that in addition to Vinculin multiple alternative force-sensitive connections between α-catenin and the actin cytoskeleton exist (e.g. through Afadin), in our opinion it is likely that these different connections might act redundantly during development, and there might thus be adaptation to the absence of Vinculin. We have now devoted a separate paragraph in the discussion to this:

In addition to Vinculin, α-catenin can recruit other actin-binding proteins (e.g. Afadin; (Sawyer et al., 2009; Matsuzawa et al., 2018)) and binds F-actin directly through a catch-bond interaction (Buckley et al., 2014; Ishiyama et al., 2018). It will be interesting to explore to what extent these alternative connections between the cadherin complex and actin network are regulated in mitotic cells. Importantly, alternative actin-associated proteins recruited to α-catenin may act redundantly with Vinculin in reinforcing mitotic cell-cell junctions, which could explain the relatively mild phenotype of selectively losing Vinculin in mice (Xu et al., 1998) and flies (Alatortsev et al., 1997).
January 25, 2021

RE: JCB Manuscript #202001042R-A

Dr. Martijn Gloerich
University Medical Center Utrecht
Universiteitsweg 100
Utrecht 3584CG
Netherlands

Dear Dr. Gloerich:

Thank you for submitting your revised manuscript entitled "An asymmetric junctional mechanoresponse coordinates mitotic rounding with epithelial integrity". The paper has been seen again by one of the original reviewers and by the editors. Unfortunately, original reviewer #1 was unable to re-review the paper. In any case, as you will see, reviewer #2 now recommends publication and thus, we would be happy to publish your paper in JCB pending some final revisions of the content and necessary to meet our formatting guidelines (see details below).

**There is one point that we feel requires a minor revision: the intestinal organoid experiments are not described in the Results section but only briefly mentioned in the Discussion. These experiments should be described in greater detail in the proper section. Please be sure to include a rebuttal statement which outlines how this issue was addressed in the final revision.**

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

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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.**

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2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis, including cropped gels. Although you list the size of the proteins of interest in the labeling of your blots in figures 3F, S2B, and S3E, you must still provide molecular weight marker indicator(s) in these figure panels.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and
methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), 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."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) 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.

6) 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
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7) 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.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. While you currently provide a list of the supplementary figures and videos, this section should be presented in paragraph form and include brief descriptions of each figure and video (see other recently published JCB papers for examples of this if these instructions are not clear).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.
Reviewer #2 (Comments to the Authors (Required)):

Thorough/scholarly and fully responsive rebuttal-- many new experiments that addressed all of my issues regarding better images (i.e., z-stack-images for clarity). Authors also well addressed Reviewer #1’s claim that the study was largely descriptive. I think the multi-photon junction ablation experiments are an important addition-- and address questions that existing tension sensors cannot.