Calcium signaling mediates a biphasic mechanoadaptive response of endothelial cells to cyclic mechanical stretch

Yekaterina Miroshnikova, Sandra Manet, Xinping Li, Sara Wickström, Eva Faurobert, and Corinne Albiges-Rizo

Corresponding author(s): Yekaterina Miroshnikova, University of Helsinki

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E21-03-0106
TITLE: Calcium signaling mediates a biphasic mechanoadaptive response of endothelial cells to cyclic mechanical stretch

Dear Kate and Corinne,

Thank you for submitting your manuscript for our consideration. It has now been seen by two reviewers, whose comments follow below.

As you can see, both reviewers are supportive. They find the work interesting and largely convincing (as do I). They do have some suggestions for improvement, so I'd invite you to consider these in a revision. Most of these issues could probably be handled with a judicious clarification in the text, although there are some controls (e.g. normalization of alpha-18 staining) that may require further experiments. I'd also ask you to clarify the localization of the biotin ligase domain - something noted by Reviewer 2 - as it would surprising to find these hits if it were placed in the extracellular domain.

To help us quickly identify the changes in your MS, it would be great if you could also include a version with the changes highlighted in a different colour.

We look forward to seeing your revision soon.

Best,

Alpha

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Miroshnikova,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is

accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The manuscript by Miroshnikova et al. reveals that endothelial cells (ECs), when exposed to cyclic biaxial stretch, undergo intriguing cellular processes in order to adapt to this type of physical strain. The authors have performed a very thorough analysis to identify molecular pathways that control this mechanical, using complementary techniques to support the data. The authors uncover two temporally separated responses. First, a calcium influx by the mechanosensitive Piezo1 channel triggers RhoA activation and increases actomyosin activity which leads to irregular cell-cell junctions. Subsequently, phosphorylation of filamins increases monolayer stiffness and allows for ECs to resist prolonged stretch.

I really like the concept of this study and the methods used. However, the methods of quantitation and some of the conclusions drawn require clarification prior to publication. Major and minor comments are as follows:

1. To assist readers, the manuscript would benefit from a schematic representation of the methodology used to induce "cyclic biaxial mechanical stretch" upon the EC monolayer.

2. The authors have used "Remodelled VE- cadherin" as a major read-out for the EC response.

However, changes in VE-cadherin patterning are not clear in all Figures, high magnification images of representative junctions would help the reader to interpret VE-cadherin remodeling. Indeed, in Fig 1, VE- cadherin is more irregular upon stretch and is expressed in a zipper-like pattern. In Fig 1C and D, the "Remodeled VE-cadherin" nicely correlates with Fig 1B and D - pMLC2 upregulation.
In Fig 5C-D, pMLC2 is still induced in the Piezo1 KD cells, yet the quantification of "Remodeled VE-cadherin" reveals a rescue of the irregular phenotype. Can the authors explain in more detail what the cellular response of Piezo1 KD cells under stretch? Furthermore, in Fig 4C-D, Fig 5A-B and Fig 6E-F, the authors quantify "Remodeled VE- cadherin" but the images represent B-catenin straining, not VE-cad.

3. It is not entirely clear to me if the authors suggest that Piezo1 would be the main route by which calcium enters the cells when stretched? There is a short decrease in Piezo 1 at the 30 min time-point, however afterwards levels return back to normal and the cells are still stretched so how do calcium levels drop so dramatically when presumably the stretch-response of Piezo1, once re-expressed, could still be triggered.

4. The authors identify 20-30 proteins that are more abundant at junctions when ECs are exposed to stretch. One of these is Vinculin and this data confirms really nicely previous studies showing that Vinculin is recruited to junctions when under high tension. In Supp Fig 1A this is examined by immunofluorescence analysis of Vinculin and in Supp 1B Vinculin intensity at junctions is further quantified. I believe also here the images could be improved to show co-localisation with VE-cadherin at high res because I find it very difficult to appreciate from the current data. Upregulation of Vinculin at Focal adhesions is most prominent.

5. In Fig 4C-D the authors have employed chemical inhibition studies to functionally analyse the consequences of inhibiting RhoA (Y-27632) and Rac-PAK (IPA3) pathways. Have the authors examined pMLC expression for this experiment? The Y-27632 treated ECs enlarge when stretched. However, the cell-cell junctions are intact and gaps do not appear in the EC monolayer. How do the authors suggest this translates to EC coping with in vivo cyclic stretch? Could cellular enlargement also be a mechanism by which vessel integrity is maintained or would this enlargement lead to compromised vessel function (bleedings)?

6. In Fig 6D, the measurements of 0% siFilA and 0% siFilB are not included but would definitely be informative.

7. In Fig 6E, the junctions of FilA and FilB KD cells are more irregular at baseline (0%CNL). Also, the KD cells are larger and do not form a tight monolayer. The authors fail to mention these differences and instead continue to show that the cells fail to adapt after 3h of stretch, quantified by "Remodelled VE-cadherin". However, the junctions were not normal in the first place which suggests that the ECs capability to respond to this methodology of stretching was not going to be comparable to control ECs.

Minor comments:
1. In Supp Fig 4C the labels of Piezo1 and GAPDH are missing.

2. In Fig 4F it is not clear whether a confluent monolayer or sparse ECs are imaged.

3. Since WB analysis of Piezo1 has been shown in Fig 5E, it would be good to also use the WB analysis to assess Piezo1 KD, to complement Piezo1 mRNA levels in Supp Fig.

Reviewer #2 (Remarks to the Author):

With great interest I have evaluated the manuscript by Miroshnikova and colleagues addressing the responses of the endothelium to cyclic stretch. Overall the study addresses an interesting research question and includes various new insights that will be of interest for the field. The key finding is that the endothelial cells first respond to stretch via Piezo, subsequently remodel their adhesion structures and over time change the organization of the cytoskeleton via filamins. It should be noted that the conclusions are partially based on elegant mass spectrometry analysis of endothelial cells that were cultured under various mechanical stretch conditions, an approach which is technically quite challenging to perform for reproducible proteomics. The manuscript contains interesting original results, and at the same time raises many new questions. For instance, to what extent does one expect the VE-cadherin interactome to change in response to stretch-induced mechano-changes? How how does this relate to the many changes that were found in the phospho-proteome? Isn't the reported change in ~30 proteins binding to VE-cadherin upon stretch potentially more important that thought? Perhaps the authors can further elaborate on their opinion on these topics in the discussion.

There were a few specific questions to address:
• The schematic in Figure 2A indicates that the biotin ligase used for BioID was fused to the N-terminus of VE-cadherin. This would mean it is tagged its the extracellular domain. Can the authors please clarify this?
• A statistical test, or cut off, of the significant changes in protein interactions in the volcano plot of Figure 2c is missing. In addition, it was not entirely clear to me whether the 30m or 3 hrs timepoint after stretching was used to in this figure panel.
• Can the authors prepare a figure referring to the 30 proteins or 33 proteins that change in the proximity of VE-cadherin in response to 30 min and 3 hrs stretch that was referred to in the results section?
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• Was there any specific type of ECM substrate used in the flexcell experiments?

Reviewer #1:

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I really like the concept of this study and the methods used. However, the methods of quantitation and some of the conclusions drawn require clarification prior to publication. Major and minor comments are as follows:

We thank the reviewer for this positive assessment of our work and the expert comments and suggestions that helped us to further improve our manuscript.

1. To assist readers, the manuscript would benefit from a schematic representation of the methodology used to induce "cyclic biaxial mechanical stretch" upon the EC monolayer.

We thank the reviewer for this suggestion and agree that it will be useful. We have amended the schematic in Fig. 1A to better describe the method of stretching.

2. The authors have used "Remodelled VE- cadherin" as a major read-out for the EC response. However, changes in VE-cadherin patterning are not clear in all Figures, high magnification images of representative junctions would help the reader to interpret VE-cadherin remodeling. Indeed, in Fig 1, VE- cadherin is more irregular upon stretch and is expressed in a zipper-like pattern. In Fig 1C and D, the "Remodeled VE-cadherin" nicely correlates with Fig 1B and D - pMLC2 upregulation.

We appreciate this criticism and have now included high magnification images of junctions in all analyses to better highlight the changes.

In Fig 5C-D, pMLC2 is still induced in the Piezo1 KD cells, yet the quantification of "Remodeled VE-cadherin" reveals a rescue of the irregular phenotype. Can the authors explain in more detail what the cellular response of Piezo1 KD cells under stretch?

We thank the reviewer for pointing this out. There is indeed a moderate increase in pMLC2 in Piezo1 KD cells in response to stretch, but this increase is much less profound than in the siCNL HUVEC monolayers. To clarify this, we have now included a quantification of pMLC2 in Figure 5D. Consistently, the junctions appear to be under some tension also in the Piezo1 KD cells at 30 minutes of stretch, albeit to a much lower degree, as we do not observe unzipping of the junctions as happens in controls. These data indicate to us that Piezo1 is required for full junctional remodeling, but either due to involvement of other channels or incompleteness of transient siRNA knockdown, some effect of stretch remains. We have edited the discussion section on p. 10 to avoid overstatement and write: “We observe that Piezo1-mediated Ca2+, activated by cyclic stretch as expected, is involved in junction remodeling.”

Furthermore, in Fig 4C-D, Fig 5A-B and Fig 6E-F, the authors quantify "Remodeled VE-cadherin" but the images represent B-catenin straining, not VE-cad.

We thank the reviewer for pointing this out. We have changed the axis labeling to “Remodeled AJs” throughout the manuscript and further clarified in each figure legend whether we have quantified beta-catenin or VE-cadherin as a proxy for adherens junctions (AJ).

3. It is not entirely clear to me if the authors suggest that Piezo1 would be the main route by which calcium enters the cells when stretched? There is a short decrease in Piezo 1 at the 30 min time-point, however afterwards levels return back to normal and the cells are still stretched so how do calcium levels drop so dramatically when presumably the stretch-response of Piezo1, once re-expressed, could still be triggered.

We realize that we have not been sufficiently clear on our interpretation of the data. Indeed, Piezo1 protein levels return to baseline at 60 min, yet intracellular calcium is no longer triggered. As Piezo1 is known to be gated by the actomyosin cortex (Cox et al., Nat Commun 2016. PMID: 26785635), i.e. the stiffer the cortex the higher the threshold for Piezo1 activation, we predict that the increased cortical stiffening driven by Filamin activation, elevates the threshold by which Piezo1 is activated in the mechanically reinforced endothelial monolayers, explaining why calcium influx is no longer efficiently triggered. We have clarified this interpretation of the findings in the results section on p. 8 and discussion section p. 11 where we state “Our data indicates that the FilaminA-mediated cortical stiffening persists beyond the peak and eventual decline in myosin phosphorylation, indicating that regulation of the cortical F-actin architecture through Filamin actin crosslinking activity, independent of contractility, is likely to play a key role in mechanically reinforcing the cytoskeleton for efficient mechanoadaptation. As the sensitivity of stretch-induced ion channels to the mean stress in the cortex can be

modified by alterations in scaffold proteins (Cox *et al.*, 2016), we speculate that this increase in cortical stiffness elevates the threshold for Piezo1 activation, and this desensitization could prevent further calcium influx despite the normalization of Piezo1 protein levels in these mechanically adapted EC monolayers.

4. The authors identify 20-30 proteins that are more abundant at junctions when ECs are exposed to stretch. One of these is Vinculin and this data confirms really nicely previous studies showing that Vinculin is recruited to junctions when under high tension. In Supp Fig 1A this is examined by immunofluorescence analysis of Vinculin and in Supp 1B Vinculin intensity at junctions is further quantified. I believe also here the images could be improved to show co-localisation with VE-cadherin at high res because I find it very difficult to appreciate from the current data. Upregulation of Vinculin at Focal adhesions is most prominent.

We appreciate this feedback and have included high magnification images to better show co-localization of VE-Cadherin and Vinculin in Supplementary Figure 1A. We have also added seminal references to previous work showing that vinculin is recruited to AJ under tension (PMID: 22391038 and PMID: 20584916) on page 4.

5. In Fig 4C-D the authors have employed chemical inhibition studies to functionally analyse the consequences of inhibiting RhoA (Y-27632) and Rac-PAK (IPA3) pathways. Have the authors examined pMLC expression for this experiment? The Y-27632 treated ECs enlarge when stretched. However, the cell-cell junctions are intact and gaps do not appear in the EC monolayer. How do the authors suggest this translates to EC coping with in vivo cyclic stretch? Could cellular enlargement also be a mechanism by which vessel integrity is maintained or would this enlargement lead to compromised vessel function (bleedings)?

Indeed we postulate that increased contractility through RhoA functions to resist cell deformation, leading to increased tension at junctions. Thus, it is further reasonable to hypothesize that when actomyosin contractility (and thus pMLC2) is blocked using the Rock inhibitor Y27632, cells would lose the ability to resist stretch-induced deformation, which might compromise cell functions. To address this, we have now included a quantification of cell spread area in control and Y-27632-treated HUVEC monolayers (new Supplemental Figure 3C) showing an increase in cell spread area with the Y27632 treatment. We also included a discussion on this topic on page 6 of the manuscript. We have also included images of pMLC2 in non-stretched HUVEC monolayers showing that, as expected Y27632 prevents pMLC2 whereas Rac-PAK inhibition using IPA increases pMLC2 (new Supplemental Figure 3E).

6. In Fig 6D, the measurements of 0% siFilA and 0% siFilB are not included but would definitely be informative.

We agree with the reviewer and we have added this data as new Supplementary Figure 5E.

7. In Fig 6E, the junctions of FilA and FilB KD cells are more irregular at baseline (0%CNL). Also, the KD cells are larger and do not form a tight monolayer. The authors fail to mention these differences and instead continue to show that the cells fail to adapt after 3h of stretch, quantified by "Remodelled VE-cadherin". However, the junctions were not normal in the first place which suggests that the ECs capability to respond to this methodology of stretching was not going to be comparable to control ECs.

We agree with the reviewer that the junctions are affected in the Filamin KD cells already in the absence of stretch. We have amended the description of the phenotype in the results section on page 9 to clarify this. As we further agree that this steady state phenotype might affect the response of Filamin-depleted cells, we have edited the conclusion to state that depletion of Filamins affects the ability of cells to adapt (rather than respond) to stretch.

Minor comments:
1. In Supp Fig 4C the labels of Piezo1 and GAPDH are missing.

We apologize for this omission and have added the labels.

2. In Fig 4F it is not clear whether a confluent monolayer or sparse ECs are imaged.

We have added the information that a confluent monolayer was imaged. As we used transient transfection, not all cells are labeled.

3. Since WB analysis of Piezo1 has been shown in Fig 5E, it would be good to also use the WB analysis to assess Piezo1 KD, to complement Piezo1 mRNA levels in Supp Fig.

We have added the requested WB analysis of Piezo1 protein levels to Supplemental Figure 4A.

Reviewer #2:

With great interest I have evaluated the manuscript by Miroshnikova and colleagues addressing the responses of the endothelium to cyclic stretch. Overall the study addresses an interesting research question and includes various new insights that will be of interest for the field. The key finding is that the endothelial cells first respond to stretch via Piezo, subsequently remodel their adhesion structures and over time change the organization of the cytoskeleton via filamins. It should be noted that the conclusions are partially based on elegant mass spectrometry analysis of endothelial cells that were cultured under various mechanical stretch conditions, an approach which is technically quite challenging to perform for reproducible proteomics. The manuscript contains interesting original results, and at the same time raises many new questions. For instance, to what extent does one expect the VE-cadherin interactome to change in response to stretch-induced mechano-changes? How how does this relate to the many changes that were found in the phospho-proteome? Isn't the reported change in ~30 proteins binding to VE-cadherin upon stretch potentially more important that thought? Perhaps the authors can further elaborate on their opinion on these topics in the discussion.

We thank the reviewer for this positive evaluation of our work and for the expert comments and suggestions that helped us to further improve this study.

There were a few specific questions to address:
• The schematic in Figure 2A indicates that the biotin ligase used for BioID was fused to the N-terminus of VE-cadherin. This would mean it is tagged its the extracellular domain. Can the authors please clarify this?

We thank the reviewer for pointing out the mistake in the schematic, indeed the BioID was fused into the intracellular C-terminus. This has been corrected in new Figure 2A.

• A statistical test, or cut off, of the significant changes in protein interactions in the volcano plot of Figure 2c is missing. In addition, it was not entirely clear to me whether the 30m or 3 hrs timepoint after stretching was used to in this figure panel.

We have added a dotted line to depict the padj cutoff of 0.05 as well as added the information on the statistical analyses in the figure legend. We have further clarified in the results section on page 4 that the volcano plot was comparing BioID alone to VE-Cadherin BioID without stretch to identify the interactome in basal conditions.

• Can the authors prepare a figure referring to the 30 proteins or 33 proteins that change in the proximity of VE-cadherin in response to 30 min and 3 hrs stretch that was referred to in the results section?

We thank the reviewer for this suggestion. We have now prepared an additional panel depicting the main functional protein groups that are changed in the proximity of VE-Cadherin at 30min and 3h of stretch (new Supplementary Fig. C)

• There were a few small errors in the Y-axis legen of Supplementary Figure 1.

We thank the reviewer for pointing out these mistakes that has now been corrected.

• In Figure 1E-F endothelial junction remodeling was analysed through staining with the alpha18 antibody that recognizes a tension sensitive domain in alpha-catenin. The authors conclude that there is an increase in intensity of alpha18 signal upon cyclic stretch of the monolayer. Potentially, the stretch induced remodeling results in a general increase of junctional proteins at the remodeling contact sites. It would be good to control for overall presence of VE-cadherin or one of the catenin proteins to normalize the alpha18 staining to.

We appreciate this criticism and have now controlled the alpha18 quantification by normalizing it to the intensity of VE Cadherin staining (new Figure 1F).

• Figure 4G: it is not clear what the kymographs reflect or emhasize in terms of junction turnover upon ionomycin treatments

We agree that the information content of the kymograph is low and have removed this panel from the manuscript.

• Was there any specific type of ECM substrate used in the flexcell experiments?

The elastomers were coated with 20 microg/mL Fibronectin in all experiments. We have clarified this in the methods section.

RE: Manuscript #E21-03-0106R
TITLE: "Calcium signaling mediates a biphasic mechanoadaptive response of endothelial cells to cyclic mechanical stretch"

Dear Kate,

Thank you for sending us your revised MS. It has been seen again by the reviewers who support all your changes.

Since everything seems to be in order, I'm delighted to accept it for MBoC.

Best wishes,

Alpha

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Miroshnikova:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Your paper is among those chosen by the Editorial Board for Highlights from MBoC. Hightlights from MBoC appears in the ASCB Newsletter and highlights the important articles from the most recent issue of MBoC.

All Highlights papers are also considered for the MBoC Paper of the Year. In order to be eligible for this award, however, the first author of the paper must be a student or postdoc. Please email me to indicate if this paper is eligible for Paper of the Year.

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www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

As requested, the revised manuscript by Miroshnikova et al. addresses the concerns that were raised on the initial version. They responded positively with a range of new experiments and more extensive quantification of data. The authors have also addressed some concerns satisfactory in the text by more elaborate discussion of their results. I therefore support publication of this revised work in Mol Biol of the Cell.

Reviewer #2 (Remarks to the Author):

The authors have addressed all of the remaining questions. Congratulations to the authors for this very elegant manuscript.