JCB

Effector-mediated ERM activation locally inhibits RhoA activity to shape the apical cell domain

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September 29, 2020

Re: JCB manuscript #202007146

Dr. Anthony Bretscher
Cornell University
Department of Molecular Biology and Genetics Weill Institute for Cell and Molecular Biology 257 Weill Hall
Ithaca, NY 14853

Dear Tony,

Thank you for submitting your manuscript entitled "RhoA effectors LOK/SLK activate ERM proteins to locally inhibit RhoA and define apical morphology." Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

As you can see from these in-depth reviews from three leading expert peer reviewers in the field, this manuscript was felt to present research of high quality, yet each of these reviewers also had substantive concerns that would require quite substantial revisions. Although we would like to invite a resubmission of this high-quality study, we have significant concerns that the added experimentation needed to raise the enthusiasm of the reviewers might not be practical for you and your laboratory. Consequently, if you feel that you can resolve the key issues in a resubmitted manuscript, we ask you and your colleagues to devise a practical revision plan to provide to JCB to help evaluate whether a resubmission will be practical.

Two of these expert reviewers note that this work represents an extension of prior conceptual advances, which would consequently require something extra to enhance enthusiasm for JCB. One key concern appears to be the question of whether the findings are generalizable, or whether they are specific to Jeg3 cells. For example, further studies with HeLa cells and ideally some other cell line with either a transient knockout or knockdown could resolve this significant concern. Although only raised by one reviewer, we agree that some quantification would be quite helpful. However, if you were to respond to the major points of all reviewers, it would not seem to us to be practical to reduce this paper in length to that of a Report, especially since this category in JCB requires a definitive study with major new conceptual advance. Instead, we would instead support a significantly expanded Article if the needed revisions are practical. If possible, immunolocalization of endogenous LOK and SLK would also be a useful advance. Although we agree with the need for some stronger establishment of mechanistic links, a key question concerning a revision would be whether any such additional analysis could be provided. In addition, there are a number of specific requests for more information or clarifications that would need to be addressed within reasonable limits for a resubmission - but evaluating where this line can be drawn in a practical sense is why we are requesting a detailed resubmission proposal if you and your colleagues are interested in performing this additional work.

We understand that the current coronavirus pandemic has disrupted research in many laboratories, so we support allowing researchers extra time. As you may know, our typical timeframe for revisions

is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit.

What is difficult with respect to this manuscript is that one of the reviewers did not support a resubmission, and the research experimentation suggested by all three expert reviewers to extend this study to make it a sufficiently major conceptual advance may be difficult. That is why we suggest using our request for a resubmission plan as an opportunity to evaluate whether you feel that the concerns can be resolved within reasonable limits. We should add that JCB Senior Editors can also provide input if there are disagreements between the expert reviewers concerning acceptability for publication to JCB.

We thank you for submitting this potentially interesting advance for the field, and we hope to hear back from you concerning your plans about a potential resubmission. Whatever you decide, we sincerely thank you for allowing us to see this very high-quality study.

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, acknowledgments, and figure legends. Count does not include materials and methods, 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.

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 or call (212) 327-8588.

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

With kind regards,

Ken

Kenneth Yamada, MD, PhD
Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

Summary
This manuscript uses Jeg-3 cells to demonstrate that the kinases LOK/SLK are Rho effectors and are the major kinases for ERM activation in the extensive apical microvilli found in this cell type. This occurs via ERM negative regulation of RhoA and activation of MLC establishing the existence of a feedback loop between Rho and ERM to maintain the distinctive structure of microvilli.
The authors use multiple approaches that strongly support the claims that are presented in the manuscript. The rescue experiments are very convincing in terms of showing specificity and the effect. The authors clearly demonstrate that Rho is upstream of LOK/SLK which are shown to be Rho effectors and that LOK/SLK activate the ERMS using several approached including Rho pulldown assays and different drug treatments. They use Crispr K/O of Ezrin, Radixin, LOK and SLK in single and double knockouts in Jeg-3 cells to conduct their experiments which is a powerful approach. The authors also demonstrate that ERM, LOK/SLK are likely involved in regulation of MLC phosphorylation and contractility in cells using western blot and live cell imaging. This work adds to the understanding of the role of ERMs in the apical domains of epithelial cells. The live cell imaging is very convincing.
This manuscript is well written and clear as presented. The data are clearly presented and overall convincing. It is an interesting analysis of the role and regulation of ERMs in microvilli.

Major concerns
The authors use Crisper K/O of Jeg-3 cells which are able to grow without ERMs or LOK/SLK albeit more slowly. This enabled the authors to dissect the mechanism of ERM in microvilli. K/O cells were not able to be obtained using other cell types. This raises the concern that the effects observed may be Jeg-3 specific effects such as the effect on the actin bundles. It would be more convincing if using knock down in other cell types that a similar (though less severe) phenotype is observed to determine the conservation of the mechanisms and phenotypes described or at least a subset of the effects in the microvilli. The conclusion that LOK and SLK are the major kinase activator of ERMs in JEG-3 cells (Ezrin and Radixin) was already demonstrated in a previous paper (Viswanatha et al 2012). This manuscript takes it a step further with the use of the phos-Tag gels but this previous work should be acknowledged in the results. The requirement in microvilli was also shown in Viswanatha et al 2012. This reduces the novelty of this claim of the paper.

The localization of LOK and SLK would be important to show by immunofluorescence to corroborate the protein interaction and drug studies. The localization of LOK-GFP and SLK-GFP was shown in Viswanatha et al 2012, which should be stated. If antibodies are available this would be an important control to demonstrate the LOK or SLK are sub-cellularly localized to the microvilli as the previous paper used transfected constructs.
Line 204 refer for Figure S3 but there is no Figure S3 and I think are referring to Figure S2B. Having immunofluorescence images in addition to the western blot are required to determine if there is any subcellular changes in localization of alpha actinin or vinculin. The overall protein levels may not change, but there could be a change in subcellular localization assuming whole cells were used.
Effect on actin in LOK/SLK K/O Jeg-3 cells very specific with an increase in actin in contractile fibers. This is different from what was observed with other mammalian cells (e.g. Bagci et al 2020 Figure 7). While this is a siRNA knockdown versus a K/O this difference should be discussed in terms of whether the effects observed are potentially Jeg-3 specific or more generalized or whether the cell type used has less microvilli for example.

Minor concerns
Line 50/51 This sentence is unclear as written based on the conclusion of the Viswanatha 2012 and in the current context of the paragraph.
Line 116 "Consistent with earlier reports" No references are provided for the earlier reports.
Line 161 Include reference for the nature and generation of the kinase dead mutant of LOK
Line 224 The reference to Figure 5A and B lanes 1 and 2 is confusing as there are no lanes present in either.
Line 355 Need the nematode reference
Line 55, 372 and 417 fly moesin should be fly Moesin http://flybase.org/reports/FBgn0011661
Line 374 It is not overly surprising that proteins in the same pathway will exhibit a similar phenotype when mutant. The common phenotype has previously been shown in the fly model (Speck 2003, Hipfner 2004). This statement should be adjusted.
Need to clarify for all figures the n values. Some are noted but others are not. Either in figure legends or in the Methods section
Would be useful to include how Licor western blots were quantified.
Figure S1, S2 then S5. Need to correct the labeling of supplemental figures.

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

Zaman et al RhoA effectors LOK/SLK activate ERM proteins to locally inhibit RhoA and define apical morphology.

Zaman et al set out to examine the relationship between the LOK/SLK kinases and substrate ERM proteins and their collective role in building the apical cell cortex. They conclude that LOK and SLK kinases are solely responsible for ERM phosphorylation and activation and conversely, that the ERMs are the major functional substrates of LOK/SLK. In mechanistic studies, they build a model wherein LOK/SLK and the ERM proteins form a local negative feedback circuit that limits RhoA activity to build the apical cortex.

This is the latest in a fine series of studies from this lab that tackle the important, complex and dynamic roles of the ERM proteins in building aspects of the cell cortex. The work is generally of high quality and the manuscript is well-written. Strengths of the study include the demonstration

that LOK/SLK are the major ERM kinases in Jeg3 cells, as in Drosophila, data (and argument) supporting the conclusion that ERMs are the key targets of LOK/SLK, and demonstration that RhoA is elevated in ERM- and LOK/SLK-deficient cells (although this has already been shown in flies and mammalian tissues). However, the mechanistic links between activated ERMs and RhoA inhibition, actin and junction organization and hierarchies, are oversimplified and need additional information and clarification.

Major points: The authors propose a model wherein LOK/SLK-activated ERMs normally limit the activity of a medioapical subpopulation of RhoA which in turn, drives changes in actomyosin organization and activity (reduced actin turnover, apical actin accumulation, defects in apical junction integrity, increased contractility) through RhoK. However, the analysis of altered actin organization and actomyosin contractility needs higher resolution and more precise tools, the requirement for RhoK needs additional support, as does the authors' assertion that activated ERMs influence RhoA activity-dependent actomyosin organization/function as opposed to actomyosin-dependent RhoA activity. It is admittedly difficult to dissect local feedback circuits without spatially localized readouts and precise tools.

Specific questions:

Fig 1: Do JEG3 cells express the other kinases that have been reported to phosphorylate the ERM C-term? The levels of Ezrin and LOK re-expression should be shown. Is the re-expressed LOK Flag-tagged as in Fig 2D, and is Ezrin also tagged (which can be problematic, as the authors themselves have pointed out)? In the single knockouts, why is there an all-or-none aspect to microvilli (ie a change in the percentage of cells that have many microvilli versus a reduction in microvilli per cell)? Could this not suggest a mechanical or biochemical threshold - and therefore less direct - mechanism?

Fig 2: D and text - it is not clear why the LOK-CTD is presented/discussed here as there seems to be no data associated with this construct?

Fig 3: (seems to include info from Figs 4 and 5): There are many mechanisms by which linear apical junction morphology can be disrupted to yield increased tortuosity. In the examples provided in this figure, the disorganization of junctional actin in the absence of ERMs versus LOK/SLK do not appear to be the same (or at the same level of confluence, junctional maturity). If the authors truly believe they are phenocopies, which seems to be a key goal of this figure, a more detailed analysis should be given. In particular, an evaluation of junctions at early and late stages of formation, quantification of the reduced junctional actin and evaluation of adherens junction proteins, since they are the major links to the actin cytoskeleton, should be included here.

What is the distribution of myolla, which is apparently expressed by these cells?

I'm not sure stress fibers is a useful term here - as opposed to concentric actin bundles that are contractile.

Fig 4 - could be merged with figure 3 and condensed.

Fig 5. The conclusion that jasplakinolide phenocopies ERM or LOK/SLK-deficiency is not made convincing by these images. Again, there is more than one way to drive junctional tortuosity and the increased apical actin in jasp-treated cells does not appear to be in the form of concentric junctional bundles as in Figure 4. This might be resolved with higher mag representative images.

Fig 6 A key result for this figure is that EzrinT567D rescues microvilli, which is stated, but not clearly shown. Why not use WAG and measure microvillus-containing cells as in Fig 1?

Fig 7 - what does Calyculin A (and blebbistatin) do to apical junctions and actin organization in these cells? Is the non-muscle myosin in the companion Fig S2B myoIIB? MyoIIa should also be evaluated. Where is the excess pMLC localized in CalyculinA-treated Ezr/Rdx-/- and LOK/SLK-/- cells mutant cells?

I think the comparator for Fig 7C is the first three lanes of 7a? If so, this is not really convincing. Also, MLCK should be ruled out. Does Y-27632 block CalyculinA induced hyperpMLC in the mutant cells?

Fig 8 'Rounding up' is an oversimplified readout of multiple integrated cellular features, in part because the cells do not appear to be rounding up as individual cells, but instead as clusters of adhering cells, which could reflect differences in junction versus cell-ECM attachment (this won't be clear from paxillin staining alone). How do the authors reconcile their conclusion about the rounding up of ERM and LOK/SLK-deficient cells with studies showing that the ERMs are required for mitotic rounding or that excess ERM activity drives increased medioapical contraction and consequent loss of junctional integrity accompanied by increased junctional actin?

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

In this manuscript, Zaman and collaborators performed a characterization of the effect of ERM proteins ezrin and radixin double KO and their specific kinase LOK/SLK in the apical cortex morphology in human epithelial cells. They found a substantial alteration in the apical domain with loss of microvilli, and junctional actin replaced by ectopic myosin-II containing apical stress-fibre-like structures. The authors attribute this phenotype to the significant function of LOK/SLK, which is to phosphorylate and activate ERMs. Finally, they found that active ERMs are negative regulators of RhoA, and propose a negative feedback loop necessary for the proper apical morphology of epithelial cells that includes the inhibition of RhoA by the activity of ERMs downstream of LOK/SLK kinases.

While these observations are quite impressive, further work is needed to validate and confirm their current hypothesis. Some of the most critical results derive from the previous observation made before by this group (Viswanatha et al. 2012; Pelaseyed et al. 2017) or described in a recent article (Bagci et al. 2020). Besides, some of the data seems preliminary. Indeed, many of the observations and conclusions relay on confocal images and western blots with no proper quantifications. Finally, I would suggest considering to concentrate the new data in a more succinct and compact article format, such as a report, which could help emphasize information that is new and extremely relevant of the current work. Therefore, several major and minor issues should be addressed before publication.

Major and minor points:

1 Figure 1: It is evident that the double KOs of Ezr / Rdx and LOK / SLK present more phenotype than the individual KOs. However, the predominant genes are Ezr and LOK in this cell type, and they are the ones that generate a more significant phenotype. The blot bands presented in panel A

are quite saturated, but it seems that Ezr KO induces an increase in Rdx expression, the authors should quantitatively analyze the expression of these proteins to determine better the potential co-regulation of these genes.

2 Figure 1: Although the confocal images are quite interesting and show the effects that the authors describe, it would also be essential to perform an electron microscopy analysis to better characterize and determine the ultrastructure of apical surface in the double KO cells lines.

3 Figure 1: The single KO of SLK does not present a significant phenotype in the formation of microvilli (figure 1C). However, the expression of LOK in the double KO of LOK / SLK is not capable of fully rescuing the phenotype in microvilli and also presents a flattened polarity phenotype that suggests that SLK could have a different or additional function in the polarity of Jeg3 cells. It might be interesting to evaluate further single LOK and SLK phenotypes in polarity (cell height, actin cytoskeleton, other polarity markers) in addition to ezrin localization described in SuppFig.1.

4 Supplementary Figures: Supplementary figures 3 and 4 are missing in the pdf containing the complete manuscript. Indeed, Fig. S3 is mentioned in the text (line 204), but not the Fig S4.

5 Figure2: In this figure is shown the effect of double KO LOK/SLK over the phosphorylation state of Ezrin in Jeg3 and Hela cells. These data extend the information previously described by the same authors (Viswanatha et al. 2012; Fig. 6C), although the new information does not seem to provide relevant data. Furthermore, the authors interpret that LOK / SLK kinases are the primary regulators of the phosphorylation and activation of ERMs in Jeg3 cells, which seems appropriate, but they suggest that this could be more general to other epithelial cells. However, they do not show any other cell type (particularly in non-tumour cells) that presents these same effects.

6 Figure 3: In line (197) describes that both double KO cells lines have an increase in the density of actin at the apical surface compared to the wt. However, the image of actin in the double LOK -/-/SLK-/- KO is remarkably different from the double Ezr-/-/Rdx-/- KO, in which a prominent actin bundling is observed. The images should be modified to include pictures that better represent the mentioned results and shown in graph 3C.

7 The title of figure 3 indicates defects in apical rigidity "..and compromises the apical rigidity of epithelial cells", but the results that describe differences in apical rigidity are analyzed and shown in figure 5.

8 Figure 4: This figure is unnecessary. The analysis of actomyosin at the apical surface could be included in figure 3 to validate further and extend the phenotype of aberrant apical cortex with prominent actin bundles. The intensity of Myo IIB should be quantified. The images of max projection presented in panel (B) of LOK -/-/SLK-/- KO the intensity of Myo IIB staining is very high and quite different from the represented in the bottom panels of 3A. This evidence should be clarified and reduced to only one panel.

9-Figure 5: The experiment of AFM is fascinating because this method allows a measure of subtle changes in rigidity at the cell surface. By contrast, the effect of the actin-modifying drugs over the tight junction remodelling and the actin cytoskeleton are apparent, but a minor contribution to the manuscript. The authors should consider reducing this data and transfer some of these results to the supplemental data. Additionally, they could also analyze the effect of LatB and Jasplakinolide in apical rigidity by AFM.

10-Figure 6: The phosphomimetic Ezrin-T567D is different to the mutant used previously by the same authors (Viswanatha et al. 2012), where the tyrosine was changed by glutamic (T567E), is there any reason for this modification, do they behave differently?
Panel B should include an image of control wt cells and KO cells lines without Ezrin to visualize the results represented in the quantification (Fig. 6D).

11- Figure 7: The western blot of pMLC in the presence of ROCK inhibitor should include the control condition, without the drug. The authors should also adequately quantify this effect.

12-Figure 8: These results should not be presented here, they do not serve to verify the model presented in the Fig. 7F, and only marginally serve to verify the effect described before on the actin and myosin cytoskeleton. It should be appropriately quantified and relocated to supplementary.

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February 4, 2021

Dan Simon, Scientific Editor
Kenneth M. Yamada, Editor
The *Journal of Cell Biology*

Re: Ms# 202007146

Dear Ken and Dan,

We are delighted to submit a carefully revised manuscript of our study entitled: "RhoA effectors LOK/SLK activate ERM proteins to locally inhibit RhoA and define apical morphology". This revision has taken a little longer than we expected, but we hope you agree that the study has been greatly enhanced by your and the referees' comments. As detailed in the attached document, we have addressed almost all of the issues. We therefore hope that this revised version will be suitable for acceptance.

With best wishes,

Tony Bretscher

Anthony Bretscher
Robert J. Appel Professor of Cellular and Molecular Biology

Authors response on JCB manuscript #202007146

Overview of major changes:

We have made significant changes to address the concerns of the editor and reviewers. A point-by-point response to each reviewer's comment is provided below with specific references to new figures/data and direct quotations added to the texts. In brief, the editor asked us to address four major concerns: 1) Are the results generalizable outside of Jeg-3 cells? 2) Can quantification of all data be provided? 3) Can the endogenous localization of LOK be reported? 4) Is it possible to provide stronger mechanistic links?

In addressing the concerns, we have added significant new data. Importantly, all the new data support and strengthen the original conclusions. Major text changes and new full sentences added are highlighted in the submitted revision. Significant changes to the main text figures include:

Figure 2A: Immunofluorescence localization of endogenous LOK.
Figure 3B: New quantification of actin junction intensity.
Figure 4A-C: Super resolution SIM imaging of actin and myosin IIB with intensity line scans.
Figure 5: Added higher magnification insets.
Figure 6A&C: Added empty vector control images.
Figure 6B: New quantification of number of cells with microvilli.
Figure 7A&B: Western blotting of phospho-myosin light (pMLC) chain under Y-27632 (ROCK inhibitor) drug treatments with quantification.
Figure 7C: Added immunofluorescence localization of pMLC under all the tested drug conditions in wildtype and knockout cells.
Figure 7D&E: New western blotting of active RhoA pulldown under Y-27632 ROCK inhibitor drug treatments with quantification. The new data in Figure 7 clarify the signalling pathway as requested.
Figure 8: New figure reporting ERM or LOK/SLK knockouts in intestinal epithelial DLD-1 cells showing junctional defects, loss of microvilli, and disruption of apical actin and hyper-activation of RhoA. Demonstrates that results from Jeg3 cells are relevant to other epithelial cells.

New additional supplemental materials listed below:

Figure S1A&C: Analysis of protein expression of ERM proteins and LOK/SLK kinases in Jeg3 single and double CRISPR cell lines.
Figure S1D: Re-expression of ezrin or LOK-GFP in knockout cell lines.
Figure S2A: Localization of E-cadherin and observation of disruption to adherens junction in knockout cells.
Figure S2B and Movie S1: Live cell imaging of GFP-ZO-1 showing the development of junctional defects.
Figure S2E: Immunofluorescence showing localization of vinculin.
Figure S3A: Immunofluorescence showing localization of myosin-IIA.
Figure S3B&C: Effect of blebbistatin on ZO-1 junctions with quantification.

Editor or reviewer comments shown in *italics.*
Original responses shown in **bold.**
New comments shown in blue.

Editors Comments:

Two of these expert reviewers note that this work represents an extension of prior conceptual advances, which would consequently require something extra to enhance enthusiasm for JCB.

For part of our paper, the reviewers are correct, but for other aspects we must respectfully disagree. Given the long-standing dispute over the relevant ERM kinases, this much more definitive study that LOK/SLK are the major players is warranted and supports earlier work. The implication that ERM proteins are THE major substrates of LOK/SLK is novel and quite remarkable. Further, consistent with this conclusion, we make the novel observation that the level of active RhoA is enhanced by loss of either LOK/SLK or ERM proteins. It should be noted that this result is obtained with endogenous RhoA, and not in an over-expression system that is commonly used. Additionally, while it was recently shown that RhoA binds SLK, we are the first to report that LOK also directly binds RhoA.

While we believe that these findings alone represent important advances, we also acknowledge the reviewers major concern regarding the need for additional novel findings. In addressing this, we will explore whether the model holds when ERMs are conditionally lost from HeLa cells, and could include the identification of an ezrin binding RhoA GAP that localizes specifically to microvilli.

One key concern appears to be the question of whether the findings are generalizable, or whether they are specific to Jeg3 cells. For example, further studies with HeLa cells and ideally some other cell line with either a transient knockout or knockdown could resolve this significant concern.

We have now generated inducible ERM and LOK/SLK knockdown HeLa cell models and can investigate whether the loss of active ERM proteins induces the same phenotypes as in Jeg-3 cells. The production of these cell lines was not trivial, and we hope the reviewers view this effort and the resulting data as a good faith attempt to generalize our findings. In our experience transient knockdowns with siRNAs do not result in the strong phenotypes seen in the knockouts as a small amount of residual ERMs obscures them.

We produced and tested conditional knockouts of LOK/SLK and ERMs in HeLa cells but found the cells' inability to form well-ordered junction's problematic for verifying our results. We therefore switched to another knockout system employing the human intestinal epithelial DLD-1 cell line. As detailed below, results with these cells are consistent with the results obtained from the Jeg3 cells.

Although only raised by one reviewer, we agree that some quantification would be quite helpful.

We will provide additional quantification and address this point in more detail below in our specific responses.

We have added new quantification of our data through the addition of subpanels to main text figures 3B, 6B, 7B&E and 8D. We have also added new quantifications of supplemental data through the addition of supplemental figures S1A and S3C. Statistical analysis of these quantification is provided in each legend matching these new subfigures and quantifications from the original submission specifying the N-value, statistical significance limits, and statistical tests used.

However, if you were to respond to the major points of all reviewers, it would not seem to us to be practical to reduce this paper in length to that of a Report, especially since this category in JCB requires a definitive study with major new conceptual advance. Instead, we would instead support a significantly expanded Article if the needed revisions are practical.

We will provide an expanded article addressing the reviewer's concerns.

If possible, immunolocalization of endogenous LOK and SLK would also be a useful advance.

We have used an excellent commercial antibody to SLK which works well in western blots but not immunofluorescence. We have produced a homemade antibody to LOK, to obtain the highest quality reagent for imaging endogenous LOK. So far, our attempts to localize endogenous LOK and SLK have not worked, but we will now explore additional fixation and visualization protocols.

We have used new fixation conditions that allow us to visualize endogenous LOK. As expected, it is enriched in microvilli. These images are now presented in Figure 2A.

Although we agree with the need for some stronger establishment of mechanistic links, a key question concerning a revision would be whether any such additional analysis could be provided.

We are currently exploring two areas in further detail related to our proposed mechanism. They are 1) the mechanism by which RhoA activity elevated, and 2) what is the contribution of ROCK (RhoK) to altered actin/myosin organization. As we mention in the discussion, the first point may be a very complex issue that could take an enormous effort to adequately resolve, and we can resolve the second issue.

Regarding the role of ROCK in our proposed mechanism, we address this concern in more detail in the specific responses to reviewer 3's comments.

In agreement with communications with the editorial team, we have not included data identifying the relevant RhoA-GAP in this manuscript. We have, however examined the pathway more carefully and can now clearly show, as detailed below, that the elevated levels of RhoA-GTP found in the knockout cells is independent of the ROCK pathway.

In addition, there are a number of specific requests for more information or clarifications that would need to be addressed within reasonable limits for a resubmission - but evaluating where this line can be drawn in a practical sense is why we are requesting a detailed resubmission proposal if you and your colleagues are interested in performing this additional work. We understand that the current coronavirus pandemic has disrupted research in many laboratories, so we support allowing researchers extra time. As you may know, our typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. What is difficult with respect to this manuscript is that one of the reviewers did not support a resubmission, and the research experimentation suggested by all three expert reviewers to extend this study to make it a sufficiently major conceptual advance may be difficult. That is why we suggest using our request for a resubmission plan as an opportunity to evaluate whether you feel that the concerns can be resolved within reasonable limits. We should add that JCB Senior Editors can also provide input if there are disagreements between the expert reviewers concerning acceptability for publication to JCB.

As indicated above, we believe we can address most issues within a reasonable time-frame, even given the constraints imposed by the pandemic.

The revision has taken longer than expected, but we believe the result is a stronger and substantial study.

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

Summary

This manuscript uses Jeg-3 cells to demonstrate that the kinases LOK/SLK are Rho effectors and are the major kinases for ERM activation in the extensive apical microvilli found in this cell type. This occurs via ERM negative regulation of RhoA and activation of MLC establishing the existence of a feedback loop between Rho and ERM to maintain the distinctive structure of microvilli.

Thank you for this nice summary.

The authors use multiple approaches that strongly support the claims that are presented in the manuscript. The rescue experiments are very convincing in terms of showing specificity and the effect. The authors clearly demonstrate that Rho is upstream of LOK/SLK which are shown to be Rho effectors and that LOK/SLK activate the ERMS using several approached including Rho pulldown assays and different drug treatments. They use Crispr K/O of Ezrin, Radixin, LOK and SLK in single and double knockouts in Jeg-3 cells to conduct their experiments which is a powerful approach. The authors also demonstrate that ERM, LOK/SLK are likely involved in regulation of MLC phosphorylation and contractility in cells using western blot and live cell imaging. This work adds to the understanding of the role of ERMs in the apical domains of epithelial cells. The live

cell imaging is very convincing. This manuscript is well written and clear as presented. The data are clearly presented and overall convincing. It is an interesting analysis of the role and regulation of ERMs in microvilli.

We greatly appreciate these positive comments and the evaluation that this is an interesting analysis of ERM proteins.

Major concerns

The authors use Crisper K/O of Jeg-3 cells which are able to grow without ERMs or LOK/SLK albeit more slowly. This enabled the authors to dissect the mechanism of ERM in microvilli. K/O cells were not able to be obtained using other cell types. This raises the concern that the effects observed may be Jeg-3 specific effects such as the effect on the actin bundles. It would be more convincing if using knock down in other cell types that a similar (though less severe) phenotype is observed to determine the conservation of the mechanisms and phenotypes described or at least a subset of the effects in the microvilli.

As indicated above, we have produced a conditional KO in HeLa and are already in the process of analyzing whether knockout of all active ERMs in Hela cells supports our model. We were able to produce short lived HeLa KOs of LOK/SLK and ERMs which support the major finding that LOK and SLK are the major kinases for ERMs (already presented in the existing Figure 1). We will use our newly produced conditional knockouts to explore if the same mechanism exists in HeLa cells.

We undertook several experiments using our conditional knockouts in HeLa cells. However, motile HeLa cells do not form well-ordered junctions and therefore preclude analysis of ZO-1/actin junctional defects, so we explored alternative potential cell lines.

DLD-1 cells are human carcinoma, colon epithelial cell line which form a monolayer of cells with well-ordered junctions. We were able to produce both ERM and LOK/SLK knockout using an inducible system. These cells show more dramatic junctional defects than those observed in the Jeg3 cells. As in Jeg3 cells, the ERM and LOK/SLK knockout DLD-1 cells have higher levels of endogenous RhoA-GTP. This data that supports the conclusions derived from analysis of Jeg3 cells is now presented in a new figure (Figure 8).

The conclusion that LOK and SLK are the major kinase activator of ERMs in JEG-3 cells (Ezrin and Radixin) was already demonstrated in a previous paper (Viswanatha et al 2012). This manuscript takes it a step further with the use of the phos-Tag gels but this previous work should be acknowledged in the results. The requirement in microvilli was also shown in Viswanatha et al 2012. This reduces the novelty of this claim of the paper.

We will make clear that Viswanatha et al 2012 showed that LOK/SLK are at least major kinases; however, in that publication a small fraction of ezrin (~10%) remained phosphorylated. This left two possibilities to explain the remaining 10%: 1) it was a result of incomplete knockdown, or 2) a result of the activity of an additional kinase. The current data with knockout cells is much more definitive and clarifies the issue proving, for the first time, that essentially all pERM at T567 is a result of LOK/SLK activity. As we showed in current Figure 1, this pertains to LOK/SLK knockouts in <u>both</u> Jeg-3 and HeLa cells.

While the reviewer is correct that the requirement for LOK/SLK for microvilli was previously shown, this paper's figures focus mostly on additional phenotypes only found when

active ERMs are totally absent. More specifically, the total removal of all LOK/SLK results in additional novel phenotypes (e.g. hyper contractility, junctional defects, Rho A activation, apical stiffness) not apparent in the knockdown.

Additional text in the introduction has been added in response to the reviewer's request for clarification of our previous findings. It now reads (lines 38-40):
" More recent evidence has suggested that the related LOK and SLK are major vertebrate kinase activators for ERM phosphorylation (Viswanatha et al., 2012)."

The localization of LOK and SLK would be important to show by immunofluorescence to corroborate the protein interaction and drug studies. The localization of LOK-GFP and SLK-GFP was shown in Viswanatha et al 2012, which should be stated. If antibodies are available this would be an important control to demonstrate the LOK or SLK are sub-cellularly localized to the microvilli as the previous paper used transfected constructs.

As indicated in the comments to the editor, we will enhance our efforts to localize endogenous LOK and /or SLK.

The requested immunofluorescence images are now presented as Figure 2A. We now include a sentence in the introduction about the localization of LOK-GFP and SLK_GFP (lines 41-44):
"Both LOK-GFP and SLK-GFP have been shown to target to the apical membrane where ERMs are activated (Viswanatha et al., 2012).".

Line 204 refer for Figure S3 but there is no Figure S3 and I think are referring to Figure S2B. Having immunofluorescence images in addition to the western blot are required to determine if there is any subcellular changes in localization of alpha actinin or vinculin. The overall protein levels may not change, but there could be a change in subcellular localization assuming whole cells were used.

We regret the wrong citation error. We will try to localize vinculin and α-actinin as requested.

We have expanded supplemental Figure S2E to include immunofluorescence images of vinculin as the reviewer suggests. No difference in localization of vinculin or paxillin was found between wild type and knockout cells. We were not able to obtain satisfactory images with commercial antibodies against alpha-actinin.

Effect on actin in LOK/SLK K/O Jeg-3 cells very specific with an increase in actin in contractile fibers. This is different from what was observed with other mammalian cells (e.g. Bagci et al 2020 Figure 7). While this is a siRNA knockdown versus a K/O this difference should be discussed in terms of whether the effects observed are potentially Jeg-3 specific or more generalized or whether the cell type used has less microvilli for example.

We will address the question whether the results are specific to Jeg-3 through the use of our conditional HeLa knockout cells.

Additionally, there are a few important differences between Bagci et al 2020 figure 7 and our study which we can make clear with additional text. First, as indicated by the reviewer, the Bagci paper images are of single knockdowns of LOK or SLK which allows for compensation in phosphorylation of ERMs. Second, the Bagci paper images single HeLa cells where we are describing an actin structure that occurs because of the development of forces within a monolayer of confluent cells. Third, the Bagci figure 7 provides a max projection of the cells

where the visible actin structures are dominated by the presence of stress fibers at the basal side of the cell while our images document the generation of contractile fibers specifically in the apical domain.

See above regarding conditional HeLa knockout cells. We have added a new Figure 8 to show that the results also apply to an intestinal epithelial line. Clarification of the images from Bagci et al. 2020 have been addressed by addition of the following sentence to the discussion (lines 435-438):
"In Bagci et al. the appearance of increased apical contractile fibers was not observed following the single knockdown of either LOK or SLK in concurrence with our finding that only double knockout of both kinases resulted in the most severe observed phenotypes (Figure S1)."

Minor concerns
Line 50/51 This sentence is unclear as written based on the conclusion of the Viswanatha 2012 and in the current context of the paragraph.

We have reordered the text to make our point more clear.

Line 116 "Consistent with earlier reports" No references are provided for the earlier reports.

We have added references (Bonilha et al., 1999; Pelaseyed et al., 2017). Now found on line 113.

Line 161 Include reference for the nature and generation of the kinase dead mutant of LOK

Information and references for this construct has been included in the methods (lines 517-518): " Sequences for LOK-GFP-Flag, LOK-CTD-GFP-Flag and LOK-K65R-GFP-Flag, were previously generated in the lab (Pelaseyed et al., 2017; Viswanatha et al., 2012)."

Line 224 The reference to Figure 5A and B lanes 1 and 2 is confusing as there are no lanes present in either.

We have clarified this text.

Line 355 Need the nematode reference

Thank you for identifying this error.

Line 55, 372 and 417 fly moesin should be fly
Moesin http://flybase.org/reports/FBgn0011661

We have changed our naming to match fly standards.

Line 374 It is not overly surprising that proteins in the same pathway will exhibit a similar phenotype when mutant. The common phenotype has previously been shown in the fly model (Speck 2003, Hipfner 2004). This statement should be adjusted.

We cited and explained both the Speck et al. (2003) and Hipfner et al. (2004) studies in our original submission. It is correct that the genetic data in the fly (where there is one ERM and one LOK/SLK) suggests they function in the same pathway, but the finding in our report that loss of either in mammalian cells generates very similar phenotypes is still quite remarkable. As noted in the original submission, the important Speck et al. (2004) paper found that loss of fly ERMs seems to result in enhanced Rho activity, although no mechanism was elucidated. A major

advance is our studies is that the regulation of RhoA activity by active ERMs seems to be confined to the apical region of the cell.

Need to clarify for all figures the n values. Some are noted but others are not. Either in figure legends or in the Methods section

All figures now have n values and proper statistical analysis.

Would be useful to include how Licor western blots were quantified.

We have added the following text to the methods to clarify this concern (Lines 558-561) "Bands were detected with HRP (Thermo Fisher) or infrared fluorescent secondary antibodies (Invitrogen or LI-COR Biosciences). Membranes were imaged using a scanner (Odyssey; LI-COR Biosciences). Blots imaged using HRP were imaged using a Bio-Rad ChemiDoc. Band intensities were calculated using ImageJ's gels toolkit. Graphpad/Prism was used for statistical analysis of gel band intensity quantification."

Figure S1, S2 then S5. Need to correct the labeling of supplemental figures.

We have replaced the labeling of supplemental figures.

All these issues are easily addressed.

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

Zaman et al set out to examine the relationship between the LOK/SLK kinases and substrate ERM proteins and their collective role in building the apical cell cortex. They conclude that LOK and SLK kinases are solely responsible for ERM phosphorylation and activation and conversely, that the ERMs are the major functional substrates of LOK/SLK. In mechanistic studies, they build a model wherein LOK/SLK and the ERM proteins form a local negative feedback circuit that limits RhoA activity to build the apical cortex.

Thank you for this nice summary.

This is the latest in a fine series of studies from this lab that tackle the important, complex and dynamic roles of the ERM proteins in building aspects of the cell cortex. The work is generally of high quality and the manuscript is well-written. Strengths of the study include the demonstration that LOK/SLK are the major ERM kinases in Jeg3 cells, as in Drosophila, data (and argument) supporting the conclusion that ERMs are the key targets of LOK/SLK, and demonstration that RhoA is elevated in ERM- and LOK/SLK-deficient cells (although this has already been shown in flies and mammalian tissues).

We thank the reviewer for their support of the quality of our science. There are genetic arguments that RhoA activity is probably elevated in ERM and LOK/SLK-deficient cells in the fly, but this has never been shown biochemically for the endogenous protein.

However, the mechanistic links between activated ERMs and RhoA inhibition, actin and junction organization and hierarchies, are oversimplified and need additional information and clarification. Major points: The authors propose a model wherein LOK/SLK-activated ERMs

normally limit the activity of a medioapical subpopulation of RhoA which in turn, drives changes in actomyosin organization and activity (reduced actin turnover, apical actin accumulation, defects in apical junction integrity, increased contractility) through RhoK. However, the analysis of altered actin organization and actomyosin contractility needs higher resolution and more precise tools,[….]

The Atomic Force Microscopy experiments we have performed are both a spatially localized and an exceptionally precise, high resolution tool to measure altered actin organization and myosin contractility at the apical membrane. This experiment alone was a herculean effort to address the concern the reviewer brings up regarding altered actin organization and contractility.

We now present new super-resolution SIM images of actin and myosin-IIB in Figure 4 and of actin and myosin-IIA in Figure S3. These are great improvements and provide approximately double the resolution of our previous confocal images.

[….] the requirement for RhoK needs additional support, as does the authors' assertion that activated ERMs influence RhoA activity-dependent actomyosin organization/function as opposed to actomyosin-dependent RhoA activity. It is admittedly difficult to dissect local feedback circuits without spatially localized readouts and precise tools.

We have tried to avoid approaches that do not give clear-cut answers, and untangling feedback loops is, as the review acknowledges, exceptionally difficult. For example, we have attempted to visualize active RhoA using available biosensors, but without success. In respect to RhoK, we plan to do several experiments to determine if inhibiting RhoK affects the ability of pERMs to enhance RhoA-GTP levels. These should go a long way to addressing this issue.

We have greatly expanded the data in Figure 7 relating to ROCK and its regulation of myosin light chain phosphorylation (pMLC) and the effects on active RhoA levels. In new panels Figure 7A, B we show how the level of pMLC is elevated in the knockout cells, and this effect is abrogated by inclusion of the ROCK inhibitor Y-27632. This is also true when we elevated the level of pMLC artificially by including the PP1 inhibitor calyculin A. Immunolocalization of pMLC under all these conditions shows that the changes seen biochemically correlate with changes in pMLC localization in the apical domain (new images in Figure 7C). With respect to the level of active RhoA-GTP, we showed that it is elevated in knockout cells, and in new experiments show that this is also the case in the presence of the ROCK inhibitor Y-27632 (new panels Figure 7D, E). Thus, in knockout cells, the elevation of RhoA-GTP is not dependent on ROCK. These results also revealed, unexpectedly, that ROCK itself can negatively regulate active RhoA in an ERM-independent manner.

Specific questions:

Fig 1: Do JEG3 cells express the other kinases that have been reported to phosphorylate the ERM C-term? The levels of Ezrin and LOK re-expression should be shown. Is the re-expressed LOK Flag-tagged as in Fig 2D, and is Ezrin also tagged (which can be problematic, as the authors themselves have pointed out)? In the single knockouts, why is there an all-or-none aspect to microvilli (ie a change in the percentage of cells that have many microvilli versus a reduction in microvilli per

cell)? Could this not suggest a mechanical or biochemical threshold - and therefore less direct - mechanism?

Yes, we have detected other claimed ERM kinases, including ROCK, MST4, and PKC, via RNAseq (unpublished) within Jeg3 cells.

We can provide western blots to show the level of re-expression of ezrin and LOK in rescue conditions. In Fig. 2D, this is untagged ezrin, which will now be made clear. We scored all-or-nothing for the presence of microvilli as even in the wildtype cells not all cells exhibit microvilli, and those that do, the number of microvilli is quite variable. Thus, for simplicity, we count the fraction of cells displaying microvilli. Thus, there is biological variability in the number of microvilli per cell so our data does not support a threshold mechanism.

We provide new western blots to show the level of re-expression of ezrin and LOK in rescue conditions in new Figure S1D.

To clarify that the ezrin in figure 2D is untagged the legend of figure 2 now reads:

" (D) Extracts of cells transfected with wildtype LOK or LOK mutants were collected and blotted for endogenous, untagged ezrin or phosphor-ERM. Lysates were also blotted for flag or LOK to check expression of the constructs relative to wildtype LOK."

Fig 2: D and text - it is not clear why the LOK-CTD is presented/discussed here as there seems to be no data associated with this construct?

We will remove LOK-CTD data.

This data no longer appears in the manuscript.

Fig 3: (seems to include info from Figs 4 and 5): There are many mechanisms by which linear apical junction morphology can be disrupted to yield increased tortuosity. In the examples provided in this figure, the disorganization of junctional actin in the absence of ERMs versus LOK/SLK do not appear to be the same (or at the same level of confluence, junctional maturity). If the authors truly believe they are phenocopies, which seems to be a key goal of this figure, a more detailed analysis should be given. In particular, an evaluation of junctions at early and late stages of formation, quantification of the reduced junctional actin and evaluation of adherens junction proteins, since they are the major links to the actin cytoskeleton, should be included here.

We will provide quantification of the junctional actin (line scans) and movies of tight junction (ZO1) formation over time. We will provide supplemental images of E-cadherin. Additionally, across experiments, when comparing conditions between WT vs KO cells, cells were always plated at the same time and to similar confluency.

Figures 4 and 5 have been significantly altered since original submission to address these and other concerns. We provide new, higher resolution SIM images of the apical actin and myosin structures in Figure 4A. These images show the striking similarities in phenotype between the two KO conditions. In the new figures, we now provide analysis of the junctional actin (Figure 3A,4C). We imaged the localization of GFP-ZO-1 during cell division which showed that the increased tortuosity seen in the knockout cells develops 1-2 hours after cell division with the addition of new figure panel S2B and new Movie1. We also present new data showing the localization of E-cadherin Figure S2A.

What is the distribution of myoIIa, which is apparently expressed by these cells?

We have previously imaged myoIIA and our preliminary results indicated a similar localization as myoIIB.

New images of MyoIIA with actin can be found in Figure S3A. Its localization is similar to MyoIIB (Figure 4A).

I'm not sure stress fibers is a useful term here - as opposed to concentric actin bundles that are contractile.

We will use only the term contractile actin bundles in this context.

Fig 4 - could be merged with figure 3 and condensed.

Both figures 3 and 4 will be significantly reorganized.

Figures 3 and 4 have been changed to address reviewers' concerns.

Fig 5. The conclusion that jasplakinolide phenocopies ERM or LOK/SLK-deficiency is not made convincing by these images. Again, there is more than one way to drive junctional tortuosity and the increased apical actin in jasp-treated cells does not appear to be in the form of concentric junctional bundles as in Figure 4. This might be resolved with higher mag representative images.

We will expand the images to include an enlarged visual of the junctions.

We have enlarged the representative images for Figure 5 which we believe resolves the reviewer's concern. We have also provided new SIM images at higher spatial resolution in figure 4A, C.

Fig 6 A key result for this figure is that EzrinT567D rescues microvilli, which is stated, but not clearly shown. Why not use WAG and measure microvillus-containing cells as in Fig 1?

As we have discussed previously (Viswanatha et al., 2012), restriction of microvilli to the apical surface requires ERM phosphocycling. Thus, expression of ezrinT567D does not restore the cells lacking ERM proteins to the wildtype phenotype, but generates a phenotype of ezrin-containing surface structures that are not confined to the apical surface. It is therefore not possible to count microvillus-containing cells, but it is possible to count cells with microvillus-like structures as visualized by WGA staining. We plan to do this.

This data is now presented in a revised Figure 3, in which the number of cells with microvilli-like structures in the rescue experiments is shown in Figure 3B.

Fig 7 - what does Calyculin A (and blebbistatin) do to apical junctions and actin organization in these cells? Is the non-muscle myosin in the companion Fig S2B myoIIB? MyoIIa should also be evaluated. Where is the excess pMLC localized in CalyculinA-treated Ezr/Rdx-/- and LOK/SLK-/- cells mutant cells?

We will provide images of fixed cells showing apical junctions and actin in Calyculin A and blebbistatin treated cells. The non-muscle myosin in figure S2B is not specific to myosin II isoform and is the same antibody published earlier (Bretscher & Weber 1978). Images of myoIIB in figure 4 used a different antibody (Biolegend cat # 909902). Imaging for myoIIA will be evaluated and images will be provided side by side with myoIIB. We are currently attempting to stain fixed cells for pMLC, however we have not confirmed this antibody for immunofluorescence.

We have added fluorescent images of pMLC without/with calyculin A treatment (Figure 7C), that correlate very well with the biochemical levels reported in Figure 7A, B. As noted above, high resolution SIM images of myosin-IIB are shown in Figures 4A. New myosin-IIA images are shown inFigureS3A. We have also examined the effect of blebbistatin treatment, after which wild type and knockout cells appear very similar. However, this result in complicated by the phenotypic effect of blebbistatin on wild type cells, so the data is now presented in Figure S3B.

I think the comparator for Fig 7C is the first three lanes of 7a? If so, this is not really convincing. Also, MLCK should be ruled out. Does Y-27632 block CalyculinA induced hyperpMLC in the mutant cells?

We are repeating all the experiments with the ROCK inhibitor Y-27632, and additionally testing whether it blocks elevation of pMLC in the mutant cells. We are testing if Y-27632 blocks Calyculin A induced pMLC in KO vs WT cells. We are also testing the inhibition of MLCK in this context as suggested by the reviewer.

We have repeated both our pMLC analysis and RhoA pulldown experiments with new experiments in the absence/presence of Y-27632 (Figure 7). These results make it clear that the elevation of pMLC in the knockout cells is due to enhanced ROCK activity (Figure 7A-C), and elevated RhoA-GTP in the knockout cells is independent of ROCK (Figure 7D, E). With these convincing results, we did not explore the use of an MLCK inhibitor.

Fig 8 'Rounding up' is an oversimplified readout of multiple integrated cellular features, in part because the cells do not appear to be rounding up as individual cells, but instead as clusters of adhering cells, which could reflect differences in junction versus cell-ECM attachment (this won't be clear from paxillin staining alone). How do the authors reconcile their conclusion about the rounding up of ERM and LOK/SLK-deficient cells with studies showing that the ERMs are required for mitotic rounding or that excess ERM activity drives increased medioapical contraction and consequent loss of junctional integrity accompanied by increased junctional actin?

We appreciate this comment and agree we did not discuss this adequately. The reviewer is quite right that groups of cells round up in clusters. In our collection of data in the presence of Calyculin A we have observed that cells first contract in clusters (0-1hr) (Figure 8) then later (1+2hrs) (data not shown) round as individual cells. We know that individual cells also round up, thereby eliminating the complexities associated with junctions. The role of ERMs in mitotic polarity alignment/rounding is a current avenue of study within the Bretscher lab. While Moesin deficient drosophila cells struggle to round up during mitosis (Carreno et al., 2008, Kunda et al., 2008), our mammalian cells do not show this defect to the same extent. Additionally, triple knockdown of ERMs in HeLa cells do not show defects in mitotic rounding, only in spindle orientation (Machicoane et al. 2014). Regardless, we now can provide a movie showing ERM and LOK/SLK KO cells undergoing division (ZO1-GFP staining of junctional formation). In these movies, cell rounding is clearly apparent and further defines the junction defect differences between WT and KO cells.

ZO1-GFP live cell imaging is discussed above and is now provided in Figure S2 and associated movie 1.

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

In this manuscript, Zaman and collaborators performed a characterization of the effect of ERM

proteins ezrin and radixin double KO and their specific kinase LOK/SLK in the apical cortex morphology in human epithelial cells. They found a substantial alteration in the apical domain with loss of microvilli, and junctional actin replaced by ectopic myosin-II containing apical stress-fibre-like structures. The authors attribute this phenotype to the significant function of LOK/SLK, which is to phosphorylate and activate ERMs. Finally, they found that active ERMs are negative regulators of RhoA, and propose a negative feedback loop necessary for the proper apical morphology of epithelial cells that includes the inhibition of RhoA by the activity of ERMs downstream of LOK/SLK kinases.

We appreciate this summary.

While these observations are quite impressive, further work is needed to validate and confirm their current hypothesis. Some of the most critical results derive from the previous observation made before by this group (Viswanatha et al. 2012; Pelaseyed et al. 2017) or described in a recent article (Bagci et al. 2020). Besides, some of the data seems preliminary. Indeed, many of the observations and conclusions relay on confocal images and western blots with no proper quantifications.

Although the reviewer wants more quantification, s/he is not specific about which figures. We have added additional quantification to a number of western blots and confocal images in an attempt address this.

As stated above in response to the editor we have added substantial new quantification of the data. Specifically, we have added new quantification subfigures 3B, 6B, 7B&E, 8D, S1A and S3C.

Finally, I would suggest considering to concentrate the new data in a more succinct and compact article format, such as a report, which could help emphasize information that is new and extremely relevant of the current work. Therefore, several major and minor issues should be addressed before publication.

We respectfully disagree; see comments from, and to, the editor.

Major and minor points:

1 Figure 1: It is evident that the double KOs of Ezr / Rdx and LOK / SLK present more phenotype than the individual KOs. However, the predominant genes are Ezr and LOK in this cell type, and they are the ones that generate a more significant phenotype. The blot bands presented in panel A are quite saturated, but it seems that Ezr KO induces an increase in Rdx expression, the authors should quantitatively analyze the expression of these proteins to determine better the potential co-regulation of these genes.

In multiple replicates, we have never seen an upregulation of radixin in ezrin knockout cells.

This is now quantitated and presented in new Figure S1D.

2 Figure 1: Although the confocal images are quite interesting and show the effects that the authors describe, it would also be essential to perform an electron microscopy analysis to better characterize and determine the ultrastructure of apical surface in the double KO cells lines.

Unfortunately, we do not have access to the necessary facility to do thin section electron microscopy. However, we now show AFM images of wildtype and mutant cells that show the contours of the apical domain and their more spread phenotype.

We were unable to gain access to the AFM facility in Vermont to complete this data collection due to the COVID-19 Pandemic travel restrictions.

3 Figure 1: The single KO of SLK does not present a significant phenotype in the formation of microvilli (figure 1C). However, the expression of LOK in the double KO of LOK / SLK is not capable of fully rescuing the phenotype in microvilli and also presents a flattened polarity phenotype that suggests that SLK could have a different or additional function in the polarity of Jeg3 cells. It might be interesting to evaluate further single LOK and SLK phenotypes in polarity (cell height, actin cytoskeleton, other polarity markers) in addition to ezrin localization described in SuppFig.1.

We appreciate the reviewer's curiosity about the loss of individual proteins. However, an in-depth analysis of the single knock-out cells seems to be tangential and beyond this study.

4 Supplementary Figures: Supplementary figures 3 and 4 are missing in the pdf containing the complete manuscript. Indeed, Fig. S3 is mentioned in the text (line 204), but not the Fig S4.

We apologize for these errors, they have been corrected.

5 Figure2: In this figure is shown the effect of double KO LOK/SLK over the phosphorylation state of Ezrin in Jeg3 and Hela cells. These data extend the information previously described by the same authors (Viswanatha et al. 2012; Fig. 6C), although the new information does not seem to provide relevant data. Furthermore, the authors interpret that LOK / SLK kinases are the primary regulators of the phosphorylation and activation of ERMs in Jeg3 cells, which seems appropriate, but they suggest that this could be more general to other epithelial cells. However, they do not show any other cell type (particularly in non-tumour cells) that presents these same effects.

We addressed this concern in the comments to the editor. Figure 2 shows that ERM phosphorylation is eliminated in LOK/SLK KO cells in both Jeg3 and HeLa cells. We are currently working to provide additional relevant data in an inducible KO cell line to show the effect is general across multiple epithelial cell types.

As detailed above, we now provide a new Figure 8 focusing on ERM and LOK/SLK KO in DLD-1 cells intestinal epithelial cells showing our results are also relevant in another type of polarized epithelial cell.

6 Figure 3: In line (197) describes that both double KO cells lines have an increase in the density of actin at the apical surface compared to the wt. However, the image of actin in the double LOK -/-/SLK-/- KO is remarkably different from the double Ezr-/-/Rdx-/- KO, in which a prominent actin bundling is observed. The images should be modified to include pictures that better represent the mentioned results and shown in graph 3C.

We appreciate the reviewer's suggestion and have chosen images more representative of the actin bundling phenotype.

We have replaced the original images with new higher resolution SIM images (Figure 4A).

7 The title of figure 3 indicates defects in apical rigidity "..and compromises the apical rigidity of

epithelial cells", but the results that describe differences in apical rigidity are analyzed and shown in figure 5.

We will alter the title of figure 3.

Figure 3 title now reads:
"Absence of activated ERM proteins alters cell-cell junctions.".

8 Figure 4: This figure is unnecessary. The analysis of actomyosin at the apical surface could be included in figure 3 to validate further and extend the phenotype of aberrant apical cortex with prominent actin bundles. The intensity of Myo IIB should be quantified. The images of max projection presented in panel (B) of LOK -/-/SLK-/- KO the intensity of Myo IIB staining is very high and quite different from the represented in the bottom panels of 3A. This evidence should be clarified and reduced to only one panel.

We will quantify the intensity of myoIIB. The intensity differences between panel B and A are a result of the fact that the images were taken using different microscopes employing different imaging techniques. We will reduce the result to one image panel and one intensity quantification panel as suggested by the reviewer.

This figure has been significantly reorganized and the data clarified as the reviewer suggested. The intensity analysis suggested by the reviewer on the original images presented several concerns as we could not confirm that images assembled through the Leica "Thunder" deconvolution software was appropriate for quantitative intensity analysis. Figure 4A now provides new higher resolution SIM images and a new subpanel (Figure 4B) with line scans showing a quantification of the fluorescent intensity across the prominent apical contractile fibers.

9-Figure 5:
The experiment of AFM is fascinating because this method allows a measure of subtle changes in rigidity at the cell surface. By contrast, the effect of the actin-modifying drugs over the tight junction remodelling and the actin cytoskeleton are apparent, but a minor contribution to the manuscript. The authors should consider reducing this data and transfer some of these results to the supplemental data. Additionally, they could also analyze the effect of LatB and Jasplakinolide in apical rigidity by AFM.

We appreciate the reviewers' interest in our AFM experiment. These experiments were performed at the University of Vermont to work with a specialized facility. Due to COVID-19 restrictions we are unfortunately unable to perform additional AFM experiments.
These restrictions were still in place at of the time of resubmission.

10-Figure 6: The phosphomimetic Ezrin-T567D is different to the mutant used previously by the same authors (Viswanatha et al. 2012), where the tyrosine was changed by glutamic (T567E), is there any reason for this modification, do they behave differently?
Panel B should include an image of control wt cells and KO cells lines without Ezrin to visualize the results represented in the quantification (Fig. 6D).

Yes, both these mutants have previously been published (Chambers & Bretscher 2005, Viswanatha et al., 2012) to have the same effect. We will provide the requested images to address the reviewer's comments.
This data is now presented in Figure 6A.

11- Figure 7: The western blot of pMLC in the presence of ROCK inhibitor should include the control condition, without the drug. The authors should also adequately quantify this effect.

As described above, we have presented an almost entirely new and expanded Figure 7 that addresses this and other issues.

12-Figure 8: These results should not be presented here, they do not serve to verify the model presented in the Fig. 7F, and only marginally serve to verify the effect described before on the actin and myosin cytoskeleton. It should be appropriately quantified and relocated to supplementary.

We have quantified this data and will move figure 8 to the supplement.

Multiple reviewers expressed a lack of enthusiasm for this figure and it has been moved to the supplement (Figure S5). It was not clear to us what type of quantification would be needed to satisfy the reviewer. Given its less prominent role in the manuscript we believe the movies (2-4) and images alone are sufficient.

March 3, 2021

RE: JCB Manuscript #202007146R

Dr. Anthony Bretscher
Cornell University
Department of Molecular Biology and Genetics Weill Institute for Cell and Molecular Biology 257 Weill Hall
Ithaca, NY 14853

Dear Tony,

Thank you very much for resubmitting your conscientiously revised manuscript entitled "RhoA effectors LOK/SLK activate ERM proteins to locally inhibit RhoA and define apical morphology." As you can see from the comments of the expert reviewers, they are all now supportive of eventual publication, albeit with some final minor revisions. Please examine the remaining comments carefully and use your judgment concerning the last revisions.

Please resubmit a final version promptly so that we can proceed to the next step efficiently. We look forward to receiving your revised interesting manuscript shortly.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles < 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 may have up to 10 main text figures.

3) 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. Please add molecular weight markers to Figures 2C & 7D and a scale bar to the magnified image in Figure 4C.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test

(for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The summary 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. While your current title will be appreciated by the specialists, we do not feel that it will be accessible to a broader cell biology audience. Therefore we suggest the following title: "An effector and ERM protein-mediated feedback loop controls RhoA activity in shaping apical cell domains"

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

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the

following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (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.

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 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. Please also provide a brief description.

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.

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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.

With kind regards,
Ken

Kenneth M Yamada, MD, PhD
Editor
The Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
The Journal of Cell Biology

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

This manuscript shows that LOK/SLK are the major kinases required for ERM activation in epithelial cells. They show that LOK is a Rho effector in addition to SLK and the effects on remodeling of the apical domain in ERM KO and LOK/SLK KO cells is via regulation of RhoA. This paper establishes a feedback loop between Rho and ERMs via LOK/SLK which clarifies this mechanism of ERM function in the apical domain and microvilli.
The authors have significantly improved the manuscript both in impact and scope. The addition of an alternate cell type which shows the same effects as Jeg-3 cells is a good addition. The reorganization of several figures with higher resolution images and quantification (actin line scans) are together very convincing of the effect on the knock out cells in Figures 3 and 4. Together with the AFM data this provide strong evidence of the authors claims on the effect of KO of ERM or LOK SLK on the apical junctions and actomyopsin network. The addition of new data is also strongly supportive of the authors claims. The use of drugs to show effects in multiple ways with rescue experiments are alaso very clear and supportive.
The additional movies are also useful in confirming the effects observed.
Overall the improvements to the manuscript have provided a clear and convincing story that is supported by the data provided. With minor changes as noted below would be acceptable for publication.

Points to be addressed:
This work establishes that LOK/SLK are the major kinases for activation of ERMs. The also demonstrate that Rho levels are increased with both ERM and LOK/SLK knockout. However I think that it should be noted in the manuscript that Speck et al 2003 not only show genetically that Moesin acts antagonistically to role it was also shown in that paper in LLC-PK1 cells that expression of dominant negative Ezrin lead to an increase in active Rho levels. The current manuscript clearly established the interaction between ERM, LOK/SLK and Rho using several approaches in the modeling of the apical domain which is novel and clarifies long standing questions in the field.
Line 40: define fly for first use to avoid the use of jargon : Drosophila melanogaster (fruit fly) then can use fly after that.
Line 50: requires a reference
Line 71: Bagci reference needed
Line 118: refer to Figure S1 D which is a western blot in describing restoration of microvilli. Not clear how this shows this effect although restoration of Ezrin is clear.
Line 157: Figures 2D In the Licor color panel I assume that green and red color labels are reversed. Kinase dead does not restore phosphorylation so should be a Ezrin band not a pT567 band.
Line 398: " In outline" does not fit with understanding of the rest of the sentence

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

The authors have done a better job of describing, documenting and quantifying their actin phenotype associated with ERM- or LOK/Slk-deficiency and added a bit of mechanistic information to the manuscript and I think it is now acceptable for publication.

I do think the authors may have missed an opportunity to consider their phenotype a result of shifting from a cortical actin network, containing many dynamic, ERM- and membrane-anchored nodes, to one of elongated, apical-spanning F-actin fibers that are anchored to and apply tension to, associated apical junctions. By eliminating the nodes, ERM-deficiency could eliminate the buffering capacity of a chicken-wire like actin meshwork to yield a stiffer surface. The new SIM images certainly would fit this, but it is not possible to discern whether the actin-perturbing drugs actually restore normal cortical meshwork organization in the absence of the ERMs, or eliminate the elongated apical F-actin filaments thereby relieving the mechanical stress on junctions.

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

The authors have made a commendable effort to address the questions raised by the reviewers. Some of the information is still not entirely new and could be included in the supplementary information. Some points suggested by the reviewers have not been made due to Covid-associated limitations, such as the suggested AFM and TEM experiments. Although it is compressible in these times, it reduces the novelty of the work. In sum, I am satisfied with the new revised version of the article. I think it has improved in several aspects concerning the previous version. However, a report would be a more appropriate format for this manuscript, in my opinion.