



Spectrin couples cell shape, cortical tension and Hippo signaling in retinal epithelia morphogenesis

Hua Deng, Limin Yang, Pei Wen, Huiyan Lei, Paul Blount, and Duoqia Pan

Corresponding Author(s): Duoqia Pan, UT Southwestern Medical School

Review Timeline:

Submission Date:	2019-07-03
Editorial Decision:	2019-08-12
Revision Received:	2019-11-26
Editorial Decision:	2020-01-08
Revision Received:	2020-01-15

Monitoring Editor: Ian Macara

Scientific Editor: Marie Anne O'Donnell

Transaction Report:

(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.)

DOI: <https://doi.org/10.1083/jcb.201907018>

August 12, 2019

Re: JCB manuscript #201907018

Dr. Duojia Pan
UT Southwestern Medical School
Physiology
5323 Harry Hines Blvd.
Dallas, TX 75390-9040

Dear Duojia,

Thank you for submitting your manuscript entitled "Spectrin couples cell shape, cortical tension and Hippo signaling in retinal epithelia morphogenesis". The manuscript was assessed by three reviewers with expertise in actin cytoskeleton and Hippo signaling, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

Overall, the reviewers considered that your study provides valuable new information on spectrin function and adds new insights into the regulation of Hippo activity by apical size and actomyosin tension. However, each reviewer had specific comments about the work that need to be addressed. One reviewer felt that the level of new insight into the mechanism of interactions between alpha-spectrin and the Arp2/3-based cortex was insufficient for JCB. However, after discussions, we are willing to consider a suitably revised version of the manuscript that addresses the points raised by this first round of review. Most of the specific comments seem reasonable and could be addressed by additional experiments and/or by modifications to the text. For example, Reviewer #1 did not understand how WASP-Arp2/3-assembled F-actin would be lost but uncapped F-actin would be retained in spectrin-depleted cells, and asked if the expression of Myr-WASP in the CP/spec-double RNAi cells would help clarify this. Reviewer #2 pointed out that the use of SqhEE as an activator of myosin II has recently been questioned (Vasquez et al., eLife, 2016). Other available reagents that increase myosin activity should be used to complement these data. Reviewer #3 commented on the apical size measurements, and that it would be important to quantify the apical size of primary PEC mutant for hts or beta-spectrin relative to the WT primary PEC in the same ommatidia and the total size area of ommatidia in each mutant background. If you elect to submit a revised version of the manuscript, we would require a point-by-point response to each of the referee comments, and the work will likely be sent for reevaluation to at least two of the external referees.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <http://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. Articles 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.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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 in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ian Macara, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

This manuscript by Deng et al. examines the cytoskeletal mechanisms underlying cell shape changes within tissues. Using a *Drosophila* retinal epithelial model, they show that the membrane skeleton protein spectrin plays a key role in determining tissue morphogenesis by mediating interactions between the plasma membrane and F-actin. A clever series of loss-of-function and gain-of-function manipulations indicate that spectrin localization appears to be driven by actin assembly via the Arp2/3 complex independent of adherens junctions.

Specific points:

1. I find the last section describing the interplay among WASP-Arp2/3, capping protein, F-actin, and

spectrin difficult to follow. I don't understand how WASP-Arp2/3-assembled F-actin would be lost but uncapped F-actin would be retained in spectrin-depleted cells. Would expression of Myr-WASP in the CP/spec-doubleRNAi cells help clarify this? Perhaps F-actin would still be lost because it couldn't be anchored by spectrin. But perhaps the absence of CP would allow it to be retained (and alter morphogenesis) because the +ends can bind to adducin or something else at the membrane. Does spectrin or adducin compete with capping protein for binding to the +ends of F-actin? Is the reason why the DiaCA-expressing cells in Fig.S3 have less spectrin at the membrane because Dia also binds the +ends of F-actin and its overexpression competes off adducin/spectrin? Further insight into what is going on at the +ends of actin in relation to adducin and spectrin would make the cytoskeletal take-home message much clearer.

2. In Fig.3, it would be nice if the authors specifically quantified the DE-cad-independent phalloidin staining (in addition to the DE-cad-associated phalloidin) in order to provide better comparisons of Fig.3E and 2G.

Minor points:

3. The authors could possibly move the quantification of apical areas from Fig.S1 to the main Fig.1.

4. The paper has several typos, including in the figures (e.g., palloidin, tesion, Effectivel).

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

The authors investigate the role of the spectrin cytoskeleton in maintaining cell shape, surface tension and the tension-dependent regulation of tissue growth through the Hippo pathway. The authors take advantage of the highly stereotypic cell numbers and shapes found in the *Drosophila* pupal retina to study the consequences of the partial loss of α -spectrin and some associated factors (β -spectrin, Adducin, Coracle).

Previous work has established that spectrin has an important role in regulating the Hippo pathway, that it changes the biomechanical properties of cells, and interacts and stabilizes the cortical actomyosin cytoskeleton. The present study confirms these findings, showing supernumerary cells in a α -spectrin compromised retina, and changes in cell shape associated with lower surface tension (as would be expected from removing an important component of the cortical cytoskeleton). Again, confirming previous findings (made in other fly tissues) it is shown that junctional myosin II activity is increased in α -spectrin compromised cells, leading to increases in tension that causes junctional recruitment of Ajuba and Warts. Junctional Warts is inactive leading to activation of Yorkie (*Drosophila* YAP) and proliferation.

The junctional activation of myosin II is at odds with the overall loss of surface tension (and the resulting expansion rather than contraction of the apical surface) observed as a result of spectrin depletion. Investigating this consequence of the loss of α -spectrin, the authors find that α -spectrin predominantly stabilizes the extra-junctional cortical actin cytoskeleton. Moreover, α -spectrin is required to establish a fraction of the actin cortex that depends on Arp2/3 (which promotes an actin meshwork less conducive to myosin II) rather than Formin (which promotes actin bundles that readily associate with myosin II). This specific requirement of spectrin for the Arp2/3-dependent assembly of part of the extra-junctional actin cortex is the main new insight of this study.

This work provides valuable new information into retinal development and spectrin function. However, my overall impression is that level of original insight and the mechanistic depth at which the interactions between α -spectrin and Arp2/3 based cortex has been investigated are insufficient to make this paper a good candidate for JCB.

Other comments:

- 1) The use of Sqh-EE as an activator of myosin II has recently been questioned by a study from the Adam Martin's lab (Vasquez et al., eLife, 2016). Other reagents that increase myosin activity are readily available (e.g. Rok-CA, KD of myosin phosphatase) that should be used to complement the Sph-EE data.
- 2) The experiment with the DEcad-aCat fusion protein is problematic. Since this fusion protein can rescue tissue development it is not clear why this protein would constitutively attach F-actin to the plasma membrane. Also, it is not clear whether the observed 'sporadic foci' of DEcad-aCat that are enriched in F-actin are actually at the plasma membrane associated. The logic of this experiment is questionable in my view.
- 3) Page 12. The observed increase in α -spectrin as a result to loss of Cora seems rather dramatic. I would not call this 'mild'.
- 4) Page 14. the use of the Y27632 inhibitor is problematic as it also inhibits aPKC, which could impact the observed phenotype.
- 5) Discussion and Figure 8. The model would suggest that α -spectrin compromised cells are not only wider with an enlarged apical surface but also shorter along the apico-basal axis. There is no evidence presented to support the latter. In general, it would be necessary to analyze the overall shape of the retinal cells and not just apical surface shape to develop a clearer view of the cell shape changes seen in α -spectrin compromised cells. One main consequence of the loss of spectrin seen in other epithelial cells is the loss of lateral membrane and a corresponding flattening of cells.
- 6) Page 17. "...study suggests that cell shape per se is not a determinant of ...cell proliferation...". Not sure what that even means given that there are cells of all types of shapes and sizes that can proliferate.

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

In this manuscript submitted by Deng et al. for publication to the Journal of Cell Biology, the authors extended their characterization of Spectrin function in order to understand how Spectrin restricts Myosin II activity, therein controlling Hippo pathway activity. They show that, while inhibiting alpha-Spectrin function or expressing a constitutive active form of Sqh, both lead to increased Myosin II activity and the upregulation of the Yki reporter gene ex-LacZ, they have opposite effect on maintaining the size of the apical area of primary PEC. The enlarged apical surface area of primary PEC in alpha-Spectrin-depleted ommatidia is associated with a reduction of the F-actin pools at the apical and lateral domains and of cortical tension. In contrast, the DE-cad-associated F-actin pool at AJs appears unaffected in alpha-Spectrin-depleted ommatidia and co-localized with higher amount of Jub-GFP and Wts-GFP, which are known to accumulate at AJs in response to tension. The authors then looked for the mechanism by which alpha-Spectrin could be recruited to the cell membrane. They show that this mechanism involves the Arp2/3 activator WASp, which promotes alpha-Spectrin accumulation at the cell lateral membrane through the Adducin Hts. They also show that beta-Spectrin, like alpha-Spectrin, stabilizes F-actin at the lateral membrane, and this requires its PH domain and that reducing cpa function can suppress the enlarged apical surface area due to alpha-Spectrin depletion. Based on these observations, the authors proposed that alpha-Spectrin

couples cell shape, cortical tension and Hippo signaling.

The data presented are globally of good quality and certainly bring some interesting pieces in the puzzle on how apical size and actomyosin tension controls Hippo pathway activity. I have nevertheless some comments that to my point of view would need to be clarified before publication in the Journal of Cell Biology.

Major comments

1. Apical size area: The quantification of Fig. S1, shows a significant enlargement of the apical size of primary PEC expressing alpha-specRNAi relative to the wild type primary PEC in the same ommatidia. The representative example in Fig. S1E-E' is also very clear. We could therefore expect that if alpha-Spectrin promotes apical size constriction in the primary, secondary and tertiary PEC, the apical surface area of the ommatidia in which all cells express alpha-SpecRNAi should be smaller than fully wild type ommatidia and I can hardly see a difference in Fig. 2C, 3A, 3B and S2C. The same phenotype is expected for hts mutant clones since it recruits alpha-Spectrin. In Fig. 4A (or when comparing Fig. 4F and G), the apical size area of whole ommatidia mutant for hts does not seem very different than wild type ommatidia. The same apply for beta-Spectrin in Fig. 5C. It would be important to also quantify the apical size of primary PEC mutant for hts or beta-spectrin relative to the wild type primary PEC in the same ommatidia and the total size area of ommatidia in each mutant background. This is to my point of view a critical point, as a strong message proposed by the authors is the uncoupling of cell shape and cell proliferation and to make sure that the enlargement of the apical size of primary PEC expressing alpha-specRNAi relative to the wild type primary PEC in the same ommatidia is not the consequence of non-cell autonomous effect.
2. Expanded: ex-LacZ is nuclear. As alpha-Spectrin and Myosin II activity affect cell shape, are the authors sure that the nuclei of ommatidial cells expressing SqhEE (Fig. 1D) or alpha-specRNAi (Fig. 1F) found on the same plane than those of wild type ommatidial cells? A staining with a nuclear marker would solve this issue. Expanded protein localizes mainly at the cell membrane. This does not seem to be the case in Fig. 3D.
3. F-actin: In Triton X-100 treated samples, the levels of Phalloidin staining in clones expressing alpha-specRNAi are strikingly reduced at the levels of the apical and lateral planes (Fig. 2C and D). This does not seem to be the case at the level of AJs, at least considering the levels at the membrane (Fig. 3E). DE-cad is used to mark the AJs plane (Fig. 3A). How the authors established whether they were looking at the apical or lateral planes? Did they use DE-cad as a reference?
4. Jub-GFP and Wts-GFP: I have a similar comment for the localization of Jub-GFP and Wts-GFP. How did the authors establish whether they were looking at the AJ plane? Did they use DE-cad as a reference?
5. PH requirement for beta-Spectrin localization at the cell membrane. Would the authors have access to a beta-Spectrin construct lacking the PH domain but fused to a membrane-targeting signal (Myr or other) to see if this can rescue the Phalloidin levels or defect in apical size area? Although time consuming if such construct does not exist, this could make the point clearer.
6. Cpa: I am not sure of how relevant is Fig. 7 at that point. It would be important to compare the Phalloidin membrane intensity between wild type, cpaRNAi, alpha-SpecRNAi and cpaRNAi/ alpha-specRNAi on the same graph (currently Fig. 7F and G) to evaluate whether the effect seen in cpaRNAi/ alpha-specRNAi is the sum of the phenotype of each individually.

Minor points

1. The quantification of apical size of primary PEC shown in Fig. S1 (or part of it) would be nice in the main Figure (Fig. 1).
2. Hts does not seem to be required only for the cortical localization of α -Spectrin but also to

maintain α -Spectrin levels, at least based on the confocal plane shown in Fig. 4B

3. P11 (last paragraph) "... (Fig. 4B-B' and J or a GFP...." Should be "... (Fig. 4B-B' and I) or a GFP...."

4. The data showing α -Spectrin in cora mutant clones (Fig. 5A) or ank1RNAi-expressing clones (Fig. 5B) would fit better in Supplementary Figure, as they do not bring much to the manuscript and are not discussed either. Although the authors claim that α -Spectrin shows a mild increase in ank1RNAi-expressing ommatidia, I do not see much difference.

5. P13 (line 12) "..., resulted in increased cortical spectrin level...." Should be "..., resulted in increased cortical F-actin and spectrin level...."

6. Fig. 7C is not mentioned in the text.

Dear Drs. Macara and Casadio:

Thank you very much for considering our paper entitled “Spectrin couples cell shape, cortical tension and Hippo signaling in retinal epithelia morphogenesis”. We are very pleased by the favorable reviews and editorial assessment. We wish to thank all the reviewers for their helpful comments and hope you will find the revised manuscript satisfactory.

Editor’s summary: Overall, the reviewers considered that your study provides valuable new information on spectrin function and adds new insights into the regulation of Hippo activity by apical size and actomyosin tension. However, each reviewer had specific comments about the work that need to be addressed. One reviewer felt that the level of new insight into the mechanism of interactions between alpha-spectrin and the Arp2/3-based cortex was insufficient for JCB. However, after discussions, we are willing to consider a suitably revised version of the manuscript that addresses the points raised by this first round of review. Most of the specific comments seem reasonable and could be addressed by additional experiments and/or by modifications to the text. For example, Reviewer #1 did not understand how WASP-Arp2/3-assembled F-actin would be lost but uncapped F-actin would be retained in spectrin-depleted cells, and asked if the expression of Myr-WASP in the CP/spec-double RNAi cells would help clarify this. Reviewer #2 pointed out that the use of SqhEE as an activator of myosin II has recently been questioned (Vasquez et al., eLife, 2016). Other available reagents that increase myosin activity should be used to complement these data. Reviewer #3 commented on the apical size measurements, and that it would be important to quantify the apical size of primary PEC mutant for hts or beta-spectrin relative to the WT primary PEC in the same ommatidia and the total size area of ommatidia in each mutant background. If you elect to submit a revised version of the manuscript, we would require a point-by-point response to each of the referee comments, and the work will likely be sent for reevaluation to at least two of the external referees.

--- We have addressed all the points highlighted by the editors with additional experiments. Below we provide a point-by-point response to each of the referee comments. New data/experiments are highlighted by yellow color in the revised manuscript.

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

This manuscript by Deng et al. examines the cytoskeletal mechanisms underlying cell shape changes within tissues. Using a Drosophila retinal epithelial model, they show that the membrane skeleton protein spectrin plays a key role in determining tissue morphogenesis by mediating interactions between the plasma membrane and F-actin. A clever series of loss-of-function and gain-of-function manipulations indicate that spectrin localization appears to be driven by actin assembly via the Arp2/3 complex independent of adherens junctions.

--- Thank you for the positive comments on our manuscript and helpful suggestions.

Specific points:

1. I find the last section describing the interplay among WASP-Arp2/3, capping protein, F-actin, and spectrin difficult to follow. I don't understand how WASP-Arp2/3-assembled F-actin would

be lost but uncapped F-actin would be retained in spectrin-depleted cells. Would expression of Myr-WASP in the CP/spec-doubleRNAi cells help clarify this? Perhaps F-actin would still be lost because it couldn't be anchored by spectrin. But perhaps the absence of CP would allow it to be retained (and alter morphogenesis) because the +ends can bind to adducin or something else at the membrane. Does spectrin or adducin compete with capping protein for binding to the +ends of F-actin? Is the reason why the DiaCA-expressing cells in Fig.S3 have less spectrin at the membrane because Dia also binds the +ends of F-actin and its overexpression competes off adducin/spectrin? Further insight into what is going on at the +ends of actin in relation to adducin and spectrin would make the cytoskeletal take-home message much clearer.

--- Thanks for the suggestions. Our findings that WASP-Arp2/3-assembled F-actin is lost but uncapped F-actin is retained in spectrin-depleted cells is consistent with our observation that only the former, but not the latter, pool of F-actin can directly recruit spectrin to cell cortex. It is unlikely these distinct behaviors can be simply explained by competition between adducin/spectrin and CP, since loss of CP does not lead to increased cortical spectrin. As suggested by the reviewer, we have overexpressed Myr-WASP in CP/spec-double RNAi cells. These cells still showed decreased cortical F-actin, similar to Myr-WASP overexpression in spec RNAi cells (Fig. S5 A-D), suggesting that the bulk of F-actin in these cells require spectrin to be anchored to the cell cortex. We have included this new data in the revision, which further supports the importance of WASP-Arp2/3-assembled F-actin in recruiting spectrin to cell cortex.

2. In Fig.3, it would be nice if the authors specifically quantified the DE-cad-independent phalloidin staining (in addition to the DE-cad-associated phalloidin) in order to provide better comparisons of Fig.3E and 2G.

---Thanks for the suggestion. Besides DE-cad-associated phalloidin staining at the cell junction, we have also included quantification of DE-cad-independent phalloidin staining in the apical and lateral cortices. All quantifications are presented in the same figure (Fig. 3 E).

Minor points:

3. The authors could possibly move the quantification of apical areas from Fig.S1 to the main Fig.1.

---Thanks for the suggestion. We have now moved the quantification of apical areas from Fig. S1 to Fig. 1 H.

4. The paper has several typos, including in the figures (e.g., pallloidin, tesion, Effectivel).

---Thanks for the suggestion. We have corrected all typos.

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

The authors investigate the role of the spectrin cytoskeleton in maintaining cell shape, surface tension and the tension-dependent regulation of tissue growth through the Hippo pathway. The

authors take advantage of the highly stereotypic cell numbers and shapes found in the Drosophila pupal retina to study the consequences of the partial loss of α -spectrin and some associated factors (β -spectrin, Adducin, Coracle).

Previous work has established that spectrin has an important role in regulating the Hippo pathway, that it changes the biomechanical properties of cells, and interacts and stabilizes the cortical actomyosin cytoskeleton. The present study confirms these findings, showing supernumerary cells in a α -spectrin compromised retina, and changes in cell shape associated with lower surface tension (as would be expected from removing an important component of the cortical cytoskeleton). Again, confirming previous findings (made in other fly tissues) it is shown that junctional myosin II activity is increased in α -spectrin compromised cells, leading to increases in tension that causes junctional recruitment of Ajuba and Warts. Junctional Warts is inactive leading to activation of Yorkie (Drosophila YAP) and proliferation.

The junctional activation of myosin II is at odds with the overall loss of surface tension (and the resulting expansion rather than contraction of the apical surface) observed as a result of spectrin depletion. Investigating this consequence of the loss of α -spectrin, the authors find that α -spectrin predominantly stabilizes the extra-junctional cortical actin cytoskeleton. Moreover, α -spectrin is required to establish a fraction of the actin cortex that depends on Arp2/3 (which promotes an actin meshwork less conducive to myosin II) rather than Formin (which promotes actin bundles that readily associate with myosin II). This specific requirement of spectrin for the Arp2/3-dependent assembly of part of the extra-junctional actin cortex is the main new insight of this study.

This work provides valuable new information into retinal development and spectrin function. However, my overall impression is that level of original insight and the mechanistic depth at which the interactions between α -spectrin and Arp2/3 based cortex has been investigated are insufficient to make this paper a good candidate for JCB.

Other comments:

1) The use of Sqh-EE as an activator of myosin II has recently been questioned by a study from the Adam Martin's lab (Vasquez et al., eLife, 2016). Other reagents that increase myosin activity are readily available (e.g. Rok-CA, KD of myosin phosphatase) that should be used to complement the Sph-EE data.

---Thanks for the suggestion. As suggested by the reviewer, we have expressed Rok^{CAT} to increase myosin II activity and observed similar results as Sqh^{EE}. We have included this new data in the revision (Fig. S1 A-A''' and I-I').

2) The experiment with the DEcad-aCat fusion protein is problematic. Since this fusion protein can rescue tissue development it is not clear why this protein would constitutively attach F-actin to the plasma membrane. Also, it is not clear whether the observed 'sporadic foci' of DEcad-aCat that are enriched in F-actin are actually at the plasma membrane associated. The logic of this experiment is questionable in my view.

---Thanks for the suggestion. To address this concern, we have used a different method to attach F-actin to plasma membrane. Specifically, we engineered a *UAS-mCD8-GFP-utABD* construct which fused the actin binding domain of utrophin (utABD) to transmembrane domain of CD8. Expression of this membrane-bound actin-binding fusion protein can prevent cortical F-actin from being removed by Triton X-100. We have included this new data in the revision (Fig. 2 E-E'') and removed the previous data with DEcad-aCat fusion protein.

3) Page 12. *The observed increase in α -spectrin as a result to loss of Cora seems rather dramatic. I would not call this 'mild'.*

---Thanks for the suggestion. As suggested by the reviewer, we have changed “mild” to “significant”.

4) Page 14. *the use of the Y27632 inhibitor is problematic as it also inhibits aPKC, which could impact the observed phenotype.*

--- It has been reported before that inhibition of aPKC caused apical constriction (Warner et al., JCB, 2009), which is opposite to the apical expansion we observed in Y27632-treated tissues in Fig. 7 E-E'". Therefore, the apical expansion in this experiment is unlikely caused by inhibition of aPKC by Y27632. We have considered using the well-established Myosin II inhibitor Blebbistatin to decrease cortical tension. Unfortunately, Blebbistatin does not work on *Drosophila* Myosin II (Straight et al. Science, 2003).

5) *Discussion and Figure 8. The model would suggests that α -spectrin compromised cells are not only wider with an enlarged apical surface but also shorter along the apico-basal axis. There is no evidence presented to support the latter. In general, it would be necessary to analyze the overall shape of the retinal cells and not just apical surface shape to develop a clearer view of the cell shape changes seen in α -spectrin compromised cells. One main consequence of the loss of spectrin seen in other epithelial cells is the loss of lateral membrane and a corresponding flattening of cells.*

---We have analyzed the overall cell shape along the apico-basal axis. Besides apical expansion, *α -spec* RNAi cells also show expansion of lateral area, as revealed by Dlg staining (Fig. S2 A-B''), quantified in Fig. S2 E). However, we could not detect visible changes in the level or localization of apical (Par3) and lateral (Dlg) markers. Thus, at least in pupal eyes, loss of spectrin does not lead to a significant loss of lateral membrane.

6) Page 17. *"....study suggests that cell shape per se is not a determinant ofcell proliferation...". Not sure what that even means given that there are cells of all types of shapes and sizes that can proliferate.*

---We apologize for the confusion. We agree that cells of all types of shapes and size can proliferate, but for a given cell type, cell shape is known to have profound impact on cell proliferation (i.e., Swiss 3T3 cells of different shape). What we have shown in this study is that opposite cell shape changes (apical constriction or apical expansion) in the pupal eye can both proliferate faster due to increased Yki activity. To avoid confusion, we have now added the

phrase “in the same cell type” when we discuss the relationship between cell shape and cell proliferation.

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

In this manuscript submitted by Deng et al. for publication to the Journal of Cell Biology, the authors extended their characterization of Spectrin function in order to understand how Spectrin restricts Myosin II activity, therein controlling Hippo pathway activity. They show that, while inhibiting alpha-Spectrin function or expressing a constitutive active form of Sqh, both lead to increased Myosin II activity and the upregulation of the Yki reporter gene ex-LacZ, they have opposite effect on maintaining the size of the apical area of primary PEC. The enlarged apical surface area of primary PEC in alpha-Spectrin-depleted ommatidia is associated with a reduction of the F-actin pools at the apical and lateral domains and of cortical tension. In contrast, the DE-cad-associated F-actin pool at AJs appears unaffected in alpha-Spectrin-depleted ommatidia and co-localized with higher amount of Jub-GFP and Wts-GFP, which are known to accumulate at AJs in response to tension. The authors then looked for the mechanism by which alpha-Spectrin could be recruited to the cell membrane. They show that this mechanism involves the Arp2/3 activator WASp, which promotes alpha-Spectrin accumulation at the cell lateral membrane through the Adducin Hts. They also show that beta-Spectrin, like alpha-Spectrin, stabilizes F-actin at the lateral membrane, and this requires its PH domain and that reducing cpa function can suppress the enlarged apical surface area due to alpha-Spectrin depletion. Based on these observations, the authors proposed that alpha-Spectrin couples cell shape, cortical tension and Hippo signaling.

The data presented are globally of good quality and certainly bring some interesting pieces in the puzzle on how apical size and actomyosin tension controls Hippo pathway activity. I have nevertheless some comments that to my point of view would need to be clarified before publication in the Journal of Cell Biology.

--- Thank you for the positive assessment and helpful suggestions.

Major comments

1. Apical size area: The quantification of Fig. S1, shows a significant enlargement of the apical size of primary PEC expressing alpha-specRNAi relative to the wild type primary PEC in the same ommatidia. The representative example in Fig. S1E-E is also very clear. We could therefore expect that if alpha-Spectrin promotes apical size constriction in the primary, secondary and tertiary PEC, the apical surface area of the ommatidia in which all cells express alpha-SpecRNAi should be smaller than fully wild type ommatidia and I can hardly see a difference in Fig. 2C, 3A, 3B and S2C. The same phenotype is expected for hts mutant clones since it recruits alpha-Spectrin. In Fig. 4A (or when comparing Fig. 4F and G), the apical size area of whole ommatidia mutant for hts does not seem very different than wild type ommatidia. The same apply for beta-Spectrin in Fig. 5C. It would be important to also quantify the apical size of primary PEC mutant for hts or beta-spectrin relative to the wild type primary PEC in the same ommatidia and the total size area of ommatidia in each mutant background. This is to my point of view a critical point, as a strong message proposed by the authors is the uncoupling of

cell shape and cell proliferation and to make sure that the enlargement of the apical size of primary PEC expressing alpha-specRNAi relative to the wild type primary PEC in the same ommatidia is not the consequence of non-cell autonomous effect.

--Thanks for the suggestions. We think the reviewer meant “larger” instead of “smaller” in the sentence “*all cells express alpha-SpecRNAi should be smaller than ...*”. As suggested by the reviewer, we have now included quantification of whole mutant *alpha-specRNAi* ommatidia vs. wildtype ommatidia (Fig. S2 C-E). Indeed, both the apical area and the lateral area of whole mutant *alpha-spec* RNAi ommatidia are significantly larger than wildtype ommatidia in the same pupal eye discs. For *beta-spec^C* ommatidia, we observed enlarged lateral area but not apical area (data added as Fig. S2 F-G’, quantified in Fig. S2 H). These results are consistent with the localization of α -Spec at both apical and lateral membrane, and the localization of β -Spec at only the lateral membrane (but not apical membrane) in *Drosophila* epithelial cells (Deng et al., 2015; Lee et al., 1997).

Similar to *beta-spec^C* ommatidia, *hts^{null}* ommatidia also show enlarged lateral area but not apical area (data added as Fig. S3 C-D’ and quantified in Fig. S3 F), despite their decreased level of apical cortex F-actin (data added as Fig. S3 A-B’ and quantified in Fig. S3 E). The lack of apical enlargement in *hts^{null}* mutant cells is likely due to the remaining level of apical cortex F-actin in these mutant cells (~ 80% as compared to wildtype control), which is significantly higher than that in *alpha-spec* mutant cells (~ 60% as compared to wildtype control) (data added as Fig. S3 G). These results suggest that there are Hts-independent mechanisms that recruit spectrin to cell membrane, which explains why there was residual spectrin in *hts^{null}* mutant cells and why *hts^{null}* mutant cells have milder overgrowth phenotype than *alpha-spec* mutant cells (Fig. 4).

2. *Expanded: ex-LacZ is nuclear. As alpha-Spectrin and Myosin II activity affect cell shape, are the authors sure that the nuclei of ommatidial cells expressing SqhEE (Fig. 1D) or alpha-specRNAi (Fig. 1F) found on the same plane than those of wild type ommatidial cells? A staining with a nuclear marker would solve this issue. Expanded protein localizes mainly at the cell membrane. This does not seem to be the case in Fig. 3D.*

---Thanks for the suggestion. As suggested by the reviewer, we have included DAPI staining to indicate that the nuclei of mutant and wildtype cells were on the same plane (data added in Fig. 1 D’ and F’). Please note that throughout this paper, we always present z-projections of multiple confocal sections at a specified apical-basal position instead of a single confocal section. Fig. 3D is the compressed projection of 20 confocal images along the z axis, which makes the Ex staining appearing less enriched on the cell membrane. Individual confocal images do show clear membrane enrichment.

3. *F-actin: In Triton X-100 treated samples, the levels of Phalloidin staining in clones expressing alpha-specRNAi are strikingly reduced at the levels of the apical and lateral planes (Fig. 2C and D). This does not seem to be the case at the level of AJs, at least considering the levels at the membrane (Fig. 3E). DE-cad is used to mark the AJs plane (Fig. 3A). How the authors established whether they were looking at the apical or lateral planes? Did they use DE-cad as a reference?*

---Yes, we use DE-cad as a reference.

4. Jub-GFP and Wts-GFP: I have a similar comment for the localization of Jub-GFP and Wts-GFP. How did the authors establish whether they were looking at the AJ plane? Did they use DE-cad as a reference?

---Yes, we use DE-cad as a reference. In addition, both Jub-GFP and Wts-GFP are known to co-localize with DE-cad (Rauskolb et al., Cell, 2014).

5. PH requirement for beta-Spectrin localization at the cell membrane. Would the authors have access to a beta-Spectrin construct lacking the PH domain but fused to a membrane-targeting signal (Myr or other) to see if this can rescue the Phalloidin levels or defect in apical size area? Although time consuming if such construct does not exist, this could make the point clearer.

--- Thanks for the suggestion. As suggested by the reviewer, we engineered a *Myr-Myc-β-SpecΔPH* construct to constitutively target β-SpecΔPH to cell membrane. Expression of *Myr-Myc-β-SpecΔPH* significantly rescued the cortical F-actin level (data added as Fig. 5 D-D' and quantified in Fig. 5 B) as well as the lateral enlargement phenotype (data added as Fig. S4 D-D' and G) in *β-spec^C* mutant cells.

6. Cpa: I am not sure of how relevant is Fig. 7 at that point. It would be important to compare the Phalloidin membrane intensity between wild type, cpaRNAi, alpha-SpecRNAi and cpaRNAi/alpha-specRNAi on the same graph (currently Fig. 7F and G) to evaluate whether the effect seen in cpaRNAi/alpha-specRNAi is the sum of the phenotype of each individually.

---Thanks for the suggestion. We have now included the quantification of phalloidin membrane intensity in these genotypes on the same graph (data added as Fig. 7F).

Minor points

1. The quantification of apical size of primary PEC shown in Fig. S1 (or part of it) would be nice in the main Figure (Fig. 1).

---Thanks for the suggestion. We have now moved the quantification of apical areas from Fig. S1 to Fig. 1 H.

2. Hts does not seem to be required only for the cortical localization of α-Spectrin but also to maintain α-Spectrin levels, at least based on the confocal plane shown in Fig. 4B.

---We agree with reviewer's suggestion. It is quite possible that α-Spectrin could become unstable when not properly localized to cell cortex. This proposition is consistent with our findings that *Myc-β-SpecΔPH*, which failed to localize to cell membrane, was detected at very low levels (Fig. 5 C'), whereas *Myr-Myc-β-SpecΔPH* or *Myc-β-Spec*, which can properly localize to cell membrane, was expressed at much higher levels (Fig. 5 D' and E').

3. P11 (last paragraph) "*....(Fig. 4B-B' and J or a GFP....*" Should be "*....(Fig. 4B-B' and I) or a GFP....*"

---Thanks for the pointing this out. We have corrected the typo.

4. *The data showing α -Spectrin in cora mutant clones (Fig. 5A) or ank1RNAi-expressing clones (Fig. 5B) would fit better in Supplementary Figure, as they do not bring much to the manuscript and are not discussed either. Although the authors claim that α -Spectrin shows a mild increase in ank1RNAi-expressing ommatidia, I do not see much difference.*

---Thanks for the suggestions. We have now moved these data to Fig. S4. The reviewer is right about the normal α -Spec level in ank1RNAi-expressing ommatidia. We meant that α -Spectrin shows a mild increase in *cora*⁵ mutant ommatidia, but not in ank1RNAi-expressing ommatidia.

5. P13 (line 12) "*..., resulted in increased cortical spectrin level....*" Should be "*..., resulted in increased cortical F-actin and spectrin level....*"

---Thanks for pointing this out. We have changed the text accordingly.

6. *Fig. 7C is not mentioned in the text.*

---Thanks for the suggestion. We have now described Fig. 7C in the text.

January 8, 2020

RE: JCB Manuscript #201907018R

Dr. Duoja Pan
UT Southwestern Medical School
Physiology
5323 Harry Hines Blvd.
Dallas, TX 75390-9040

Dear Dr. Pan:

Thank you for submitting your revised manuscript entitled "Spectrin couples cell shape, cortical tension and Hippo signaling in retinal epithelia morphogenesis". We would be happy to publish your paper in JCB provided the remaining points raised by Reviewer #2 are addressed through edits to the text and pending final revisions necessary to meet our formatting guidelines (see details below).

- Provide main and supplementary text as separate, editable .doc or .docx files
- Provide figures as separate, editable files according to the instructions for authors on JCB's website, paying particular attention to the guidelines for preparing images at sufficient resolution for screening and production
- Add conflict of interest statement to Acknowledgements section
- Add author contributions

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, <http://jcb.rupress.org/submission-guidelines#revised>. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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, <http://jcb.rupress.org/fig-vid-guidelines>.
- Cover images: If you have any striking images related to this story, we would be happy to

consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

****It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.****

****The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.****

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

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.

Sincerely,

Ian Macara, Ph.D.
Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

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

The authors have improved their paper by responding for the most part appropriately to the various comments of the reviewers. As a result, the paper has been improved. My overall concern that this paper does not have sufficient novelty to make it a strong candidate for JCB has not been alleviated, though. However, I am happy to go along with the majority opinion of the other reviewers and the editor. I have some concerns directed at points made in the rebuttal and the corresponding sections of the paper.

1) Reviewer 1, point 2: 'DE-cadherin independent phalloidin' The authors do not know that. To equate E-cadherin with the apical adherens junction is a misconception often found in the literature. Ecad is generally found along the lateral membrane in epithelia, both in mammals and Drosophila, with tissue specific degrees of enrichment at the apical junction. There is much literature suggesting that Ecad stabilizes the lateral cortex. The fact that the authors do not see Ecad laterally may just mean that their staining are not the best or that Ecad is very low. E-cad could be excluded from the septate junction, but this junction does not cover the entire lateral membrane. Moreover, it has been shown that Ecad levels at a few percent of normal are functional and can support AJs and epithelial polarity. Please fix.

2) Reviewer 2, point 4: The Y compound could still interfere with aPKC only that the myosin related phenotype predominates.

3) Reviewer 2, point 4: Dlg is highly enriched at the septate junction and serves as a septate junction marker when they are present. The septate junction does not cover the entire lateral membrane of epithelial cells. So, Dlg cannot be used as a valid marker for the lateral membrane. Moreover, it is not clear how the loss of spectrin affects septate junction integrity and distribution. Again, Dlg is not an appropriate marker here. Perhaps the authors should delete these data and make clear in the discussion that they are assessing only apical cell shape but not overall cell shape or volume.

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

The authors have answered the concerns about their manuscript. To my point of view, the data are strong and bring novel and interesting knowledge on how cell shape and cortical tension control Hippo pathway activity. I therefore recommend publication in JCB.

Dear Drs. Macara and O'Donnell:

Thank you very much for your willing to publish our paper entitled “Spectrin couples cell shape, cortical tension and Hippo signaling in retinal epithelia morphogenesis”. Per your instruction, we have provided the following:

- Provided main and supplementary text as separate, editable .doc files.
- Provided figures as separate, editable files according to the instructions for authors on JCB's website.
- Added conflict of interest statement to Acknowledgements section.
- Added author contributions.

The following is our response to the Reviewer #2's comments:

1) Reviewer 1, point 2: 'DE-cadherin independent phalloidin' The authors do not know that. To equate E-cadherin with the apical adherens junction is a misconception often found in the literature. Ecad is generally found along the lateral membrane in epithelia, both in mammals and Drosophila, with tissue specific degrees of enrichment at the apical junction. There is much literature suggesting that Ecad stabilizes the lateral cortex. The fact that the authors do not see Ecad laterally may just mean that their staining are not the best or that Ecad is very low. E-cad could be excluded from the septate junction, but this junction does not cover the entire lateral membrane. Moreover, it has been shown that Ecad levels at a few percent of normal are functional and can support AJs and epithelial polarity. Please fix.

---Thanks for the suggestion. We agree with this comment. To avoid confusion, we used the term 'AJ-associated F-actin' or "non-AJ-associated cortical F-actin", instead of 'DE-cadherin dependent F-actin' or 'DE-cadherin independent F-actin' in our paper.

2) Reviewer 2, point 4: The Y compound could still interfere with aPKC only that the myosin related phenotype predominates.

--- We agree with this comment and have revised the text accordingly.

3) Reviewer 2, point 4: Dlg is highly enriched at the septate junction and serves as a septate junction marker when they are present. The septate junction does not cover the entire lateral membrane of epithelial cells. So, Dlg cannot be used as a valid marker for the lateral membrane. Moreover, it is not clear how the loss of spectrin affects septate junction integrity and distribution. Again, Dlg is not an appropriate marker here. Perhaps the authors should delete these data and make clear in the discussion that they are assessing only apical cell shape but not overall cell shape or volume.

--- The Dlg data serve to reveal the normal apical-basal polarity of spectrin mutant cells, and thus should be kept. As suggested by the reviewer, we have revised the text to indicate that we are only assessing Dlg-positive lateral area.