JCB

Sidekick dynamically rebalances contractile and protrusive forces to control tissue morphogenesis

Jacob Malin, Christian Rosa, Sergio Astigarraga, Jessica Treisman, and Victor Hatini

Corresponding Author(s): Victor Hatini, TUfts University School of Medicine

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August 15, 2021

Re: JCB manuscript #202107035

Prof. Victor Hatini
TUfts University School of Medicine
Development, Molecular & Chemical Biology (DMCB)
150 Harrison Avenue
Boston 02111

Dear Prof. Hatini,

Thank you for submitting your manuscript entitled "Sidekick dynamically rebalances contractile and protrusive forces to control tissue morphogenesis" to the Journal of Cell Biology. The manuscript has now been assessed by two expert reviewers, whose reports are appended below. As you can see from the reviews provided by these leaders in research areas spanning the elements of this paper, there was potential interest in the conclusions, but also some substantive concerns about the strength of the conclusions that would need to be resolved. After an assessment of the reviewer feedback, which varied significantly in their level of enthusiasm for this study, our editorial decision is to invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers indicated concerns about the strength of the model. It would indeed be valuable to test further the competition model by competition immunoprecipitation and colocalization analyses. In addition, the expert reviewers each listed a variety of detailed concerns with a need for clearer and more convincing data or requests for clarifications that appear to us necessary to address.

Therefore, if you feel that you can resolve the specific and general concerns of these expert peer reviewers to their satisfaction, we will be happy to send a significantly strengthened manuscript back to these expert reviewers for a final decision concerning it suitability for publication in JCB.

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, 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. 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.

As you may know, the 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. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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,

Kenneth Yamada, MD, PhD
Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

Malin and colleagues link the adhesion molecule Sidekick to the recruitment of cytoskeletal regulators during adherens junction remodeling of the developing Drosophila eye. Cell-cell contacts of the eye were previously shown to undergo repeated cycles of WRC-dependent expansion and actomyosin-dependent contraction. Sidekick mutants were known to have eye defects, and the current study shows that abnormal expansion-contraction cycles are involved. Several pieces of evidence link Sidekick to the WAVE regulatory complex (WRC), including: (1) colocalization of a WRC component with Sidekick at junctions during their cycling stage; (2) loss of junctional WRC localization in Sidekick mutants; and (3) pull-down of a WRC component by the Sidekick cytoplasmic tail in the presence of consensus sequence for WRC binding. Since this same site was implicated in Sidekick interaction with Polychaetoid, the authors compared the localization of Polychaetoid with F-actin and WRC during the expansion-contraction cycles and found that Polychaetoid enriches at tri-cellular junctions of a contact as it contracts, in contrast to accumulations of F-actin and the WRC which localize to the contact length as it expands. The authors provide evidence that Sidekick transiently accumulates at each of these sites during the contraction-expansion cycles, and in addition to being required for the WRC accumulation to contacts, Sidekick is also required for the localization of Polychaetoid to the tri-cellular junctions. Thus, a model is proposed in which Sidekick toggles between Polychaetoid-association to promote contact contraction and WRC-association to promote contact expansion. Overall, this is a very interesting finding related to the regulation of contractile and expansive actin networks at adherens junctions. Conservation of the players suggests broad relevance to tissues across animals. The work should be of interest to those studying cell-cell adhesion, actin networks, and/or tissue morphogenesis.

The paper could be improved by addressing the following points.

1. Here, SCAR was shown to be pulled down by a fusion protein of the Sidekick cytoplasmic tail. In an earlier paper by the authors, Polychaetoid was shown to bind a fusion protein of the Sidekick cytoplasmic tail. Both interactions were lost when a terminal sequence was deleted from the Sidekick cytoplasmic tail, suggesting they bind to the same site. To confirm that they bind competitively to the same site, and to exclude that the site's deletion has non-specific effects on the fusion protein of the Sidekick cytoplasmic tail, the authors could test if the SCAR pull down by the Sidekick cytoplasmic tail is reduced by additional inclusion of a protein fusion of the Polychaetoid PDZ domains, shown by the authors to also bind the Sidekick cytoplasmic tail.

2. Additional evidence for the competitive binding model could also come from testing Sidekick-WRC colocalization in a Polychaetoid mutant, or Sidekick-Polychaetoid colocalization in a WRC mutant.

3. The authors "found that deleting the C-terminal motif from HA::Sdk almost abolished its strong dominant negative effects (Fig. 4H)" and used this data as an argument that Sdk functions with the WRC. However, the authors argue elsewhere that the WRC and Polychaetoid bind to the same site of Sdk. Thus, more caution should be taken in interpreting the results of deleting the C-terminal motif from HA::Sdk.

4. In Fig 3B-F, showing additional single channel images is important for comparing the protein distributions. In particular, it is recommended to additionally show Sdk-GFP as single channel images.

5. In Fig S2C-D, the phospho-myosin staining is not convincing-the described "apicomedial pool" is not obvious in the control, nor is its loss with the Rho kinase inhibitor. This issue might be due to non-specific/variable staining (much of the signal seems to be cytoplasmic since strong nuclear exclusion is apparent). I believe the change in the Sdk-GFP distribution can still be interpreted if the phospho-myosin staining was left out.

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

Summary:

This manuscript presents very nice live-imaging data that reveals changes in actin and myosin dynamics at LC-LC boundaries when Sdk levels are modified, consequent to modified localization of Rok and WRC components. The authors show that Sdk interacts with SCAR via its C-terminal domain to recruit the WRC to LC-LC boundaries. One flaw here is that, whilst the biochemical data is good, in tissue the effect of removing Sdk on SCAR localization is weak so that Sdk is partially redundant with other mechanisms. They also provide data that Sdk recruits Pyd, but these show only a mild effect when Sdk is absent. They present a very interesting model in which Sdk alternatively recruits SCAR (to drive membrane expansion) and Pyd (to drive membrane contraction). This would very nicely explain Sdk's central role in dynamic 'pumping' of the LC-LC membrane length, although how Sdk toggles between recruiting Pyd and WRC is unknown and is key to the model. A major flaw, however, is that the effect of Sdk on the WRC and Pyd is weak. This undermines their model and the strength of this manuscript so that I'm undecided as to whether the paper is strong enough for JCB. This weakness is unsolvable for this manuscript. The revisions requested below include more thorough discussion that Sdk provides a partially redundant mechanism of WRC/Pyd recruitment (this is mentioned in their discussion but language could be modified elsewhere in the text), and there are some inconsistencies in the data that need addressing. The manuscript is well written and an enjoyable read. I hope to review the revised paper.

Regarding the Intro:
Some citations need reconsideration.
For example on Page 3 paragraph 2:
Sentence 2 - Cagan and Carthew citations are correct, but the Galy, Del Signore and Zallen citations do not relate to this sentence.
Sentence 3 - Bao and Cagan, 2005 is an odd citation choice here.
Final few sentences - if all info discussed relates to Del Signore, perhaps insert this citation earlier?

Regarding Figure 1 and associated text/supplementary data:

Broad comments on Figure 1:
1A - key could be more intuitive: a) needs more space to separate the words '2{degree sign}' 'doomed' and 'bristle' (I first read this as one label and wondered why you thought the bristles were doomed!). b) if shapes of cells in the key were taken directly from the ommatidia tracings, would be more intuitive (all shapes used are similar but not quite the same).
1D - image should be rotated ~5{degree sign} clockwise to align along the dorsal-ventral axis of the eye.
1I,J,K,M,N .... Please make these panel numbers easier to see!
1O - I'd prefer to see an image of Sdk localization that encompasses a larger eye region. The central blue and green dots placed on the 2{degree sign} and 3{degree sign} cell can easily be mistaken for sdk-GFP puncta! Your figure legend states this tissue is 42 h APF, but in the manuscript states 36 h APF.

Page 5 - the authors state that the cellular rosette phenotype is one 'that arises from defects in cell intercalation' but other defects can cause this too (eg. compromised cell survival, defects in primaries). Indeed you observe and report changes in cell survival and shape defects (which may or may not be consequent to cell position). My objection here can be resolved by stating: '... cellular rosettes, a phenotype that can arise from defects in cell intercalation'.

Page 5 - from paragraph 2 - the authors jump from discussion of intercalation (previous paragraph) to comparison of the 'pumping' dynamics of LCs that occurs after intercalation. Please make it clear to readers that these events are temporally distinct, and that the live-imaging data relates to the later events.
As always, your live imaging is very good. But I have problems with the comparison of I and J:
a) why did you chose to compare behavior of LCs located horizontally (with respect to dorsal and ventral ommatidia) in wild type tissue, with LCs located obliquely in sdk[mut]? Are the dimensions and dynamics of cells in these different positions truly identical (in wild type)? There are (usually) fewer cells in oblique positions.
b) Also, in the sdk[mut], but not wt, one cell dies. You comment on re-establishment of the new LC-LC contact in the text - why this re-establishment is needed is hard to understand for non-eye experts (remedy: point out dying cell). But importantly, please provide wt data of a similar new LC-LC contact being established so that readers can see the comparison.
c) Since the central LC is dying in the sdk[mut] tissue, which pulsing membrane are readers to compare?
d) We also have a dying cell in K but comparisons between the green LC and unmarked LC to its right can be made. If this is your intention, perhaps indicate this with an arrow head?
e) Please indicate (in the text or figure legend) the age of the retinas at the start of live-imaging. If 36 h APF, then data presented in panel O matches well. If earlier, please comment on whether Sdk localizes in the same way earlier.

Regarding Figure 2 and others:

Several superimposed white boxes (eg. Fig 2, lower panels of A, B and C; Fig 3A, B, etc.) are not easy to see (the borders of these are inconsistent - perhaps something odd happened when the pdf of the manuscript was built?).

Regarding Figure 3 and associated text/supplementary data:

It's not clear why data is presented from inconsistent ages APF - Utr, Abi at 24 h, p-Sqh at 32 h. Presentation of Scar localization at 26, 32 and 40 h does make sense as this maps changes in the localization of Sdk (great data!). Since in the text, you state that 'pumping' occurs from 24-28 h APF, analyses of p-Sqh within this window would make more sense. Analyses of Utr, Abi and p-Sqh at 26 h APF would more logically fit with the Sdk/SCAR data presented.

Regarding Figure 4 and associated text/supplementary data:

I found your comment that 'HA::Sdk overexpression promotes dispersion and degradation of WRC subunits from the cell surface' hard to follow. Why the suggestion of degradation? Surely, since HA-Sdk mis-localizes to LC-primary cell boundaries, SCAR would be mislocalized there too? But do you observe this? I can't see this consistently in the data presented. Replacing 'cell surface' to 'cell boundary' or 'cell contact' would be more precise.

Data presented in Fig4 A-B is not especially strong. A ~25% decrease of SCAR localization at LC-LC boundaries is not convincing. In panel A, a decrease in SCAR between mutant LCs can be nicely seen toward the top of the image, but the effect is negligible in mutant ommatidia at the bottom of the panel.

Did you mean to include the boxed regions of panels A and C and higher resolution (larger?) That would be helpful to readers.

However, the GST pulldown data is more convincing. I would have liked to see more GST-Sdk present in the Coomassie stain. As a matter of interest, what are the 2 lower bands below SCAR on the WB?

I would like to see - perhaps as a supplemental image - an image of SCAR localization in GMA>HA::Sdk-deltaCT.

Given the weak changes in SCAR localization in sdk mutant LCs, please soften the language to make clear that Sdk promotes SCAR localization but is partially redundant.

What is the age APF of the data presented in Figure 4A, C, E, F? E and F look to be around 36-40 h APF which, according to Fig 3E is when less membrane-associated SCAR is observed. Would the effects of GMR>Sdk not be more convincing at 28 h APF?

This is probably picky (!): Fig 4B - there's a disconnect between your use of tAJs/bAJs in this panel and 'vertices' in the text. Perhaps this could be solved by, somewhere earlier in the manuscript, clarifying how you are using these terms.

Regarding Figure 5 and associated text/supplementary data:

These data are gorgeous.

I'm confused about panels F-J: it's not completely clear whether these analyses are of Sdk at vertices only or along the entire LC-LC contact. Can you make this clear on the panels or in the figure legend? I assume that for panels C-D Sdk along the entire LC-LC contact was quantified? If this is incorrect, please make clearer.

Regarding Figure 6 and associated text/supplementary data:

Comment: GMR>RacN17 is very heavy handed, in that adherens junctions fail to form correctly and are poorly distributed in this genotype. This could account for or contribute to lack of localization of sdk-GFP in this genotype. You also state that expression of MLCK-ct , which increases tension, did not modify Sdk localization (did I misunderstand this phrase in your text as I see a difference in Fig 6?).

Comment on Figure S2 - I wonder if the strange clumping of Sdk in B indicates (direct or indirect) cis-interactions between Sdk?

Regarding Figure 7 and associated text/supplementary data:

Again, beautiful live imaging data ....

You quantify the changes in Pyd vertex localization in response to loss of Sdk in comparison to Pyd at the center of an LC-LC boundary. Could you clarify that this was because localization of Pyd along LC-LC boundaries was unaffected by Sdk? The effect looks very mild, but stronger at tAJs/bAJs between LCs rather than at LC-primary vertices. Do your analyses support this? If yes, then would you agree that there is an additional mechanism of recruitment of Pyd to LC-primary junctions that's redundant with Sdk? Stating this ahead of your comment that '... Sdk controls the timing of Pyd recruitment ...' would not undermine your conclusions.

Please include 'APF' after '26 h' (page 12, line 2). I haven't checked whether this is needed elsewhere in the manuscript.

Regarding the Discussion:

This is a very good Discussion.

December 28, 2021

We are pleased to submit a revised manuscript addressing the insightful comments of the Reviewers. By addressing these comments, we were able to improve the clarity of the manuscript and strengthen the model we developed. Below, we list Reviewers' comments in italics followed by an explanation of our revisions, starting with major concerns and moving on to minor issues. Text changes to the manuscript are highlighted in gray.

Reviewer #1 noted that given that our data show that the WRC and Pyd bind to the same C-terminal sequence motif of Sdk, we could show that they can bind competitively to this site either biochemically or genetically.

We pursued both the biochemical and genetic experiments the reviewer proposed and, although there were some technical issues described in more detail below, the results strengthen the conclusion that both Pyd and the WRC bind to the same sequence motif of Sdk. At the same time, we clarify in the manuscript that our data suggest that the binding of Pyd and the WRC to Sdk is not likely a simple competition because it is regulated temporally by contractile and protrusive forces. At high tension Sdk concentrates at vertices and binds preferentially to the WRC to promote protrusive behavior and decrease tension. At low tension, Sdk levels at vertices decrease and Sdk switches its binding preference from the WRC to Pyd to promote contractility and increase tension. In the Discussion we suggest that switching of binding partners could arise from conformational changes or clustering of Sdk proteins in response to force (page 13).

In the genetic approach suggested by the Reviewer, to determine whether Pyd can affect the interaction of the WRC with Sdk and vice versa, we examined SCAR accumulation in *pyd* mutant clones and Pyd accumulation in *SCAR* mutant clones. We have added new data showing that SCAR levels increase moderately in *pyd* mutant clones in fixed tissue. These new results are now shown in Fig. 8 and described in the last paragraph of the Results section (page 12) and in the Discussion (page 13). These results provide further evidence that both Pyd and the WRC bind to the same region in Sdk.

In contrast to the *pyd* mutant cells, which can form large clones, *SCAR* mutant clones are outcompeted by wild type cells. Therefore, it is not possible to obtain patches of mutant cells but only sporadic single cell clones that are induced relatively late in development that enable them to survive. In these single cell clones we were unable to detect a change in Pyd level. However, this may be due to the perdurance of the Pyd protein in these clones or the rescue of Pyd accumulation by the adjacent wildtype cells. Due to the difficulty in interpreting these negative data, they are not included in the current version of the manuscript. We also pursued the pull-down assays as suggested by the reviewer to determine if transfected Myc-Pyd-PDZ would outcompete the interaction of Sdk with the native SCAR protein. However, we found that the signal intensity of the Myc-Pyd-PDZ protein produced from a transfected expression plasmid, under optimal conditions, was 30-fold lower than that of the highly abundant native SCAR protein suggesting that it is unlikely to outcompete the Sdk-SCAR interaction. Therefore, we focused on the genetic tests described above to test if Pyd and the WRC bind to the same region in Sdk *in vivo*.

Reviewer #2 noted that the changes in SCAR and Pyd localization that we observed in sdk mutants were relatively small.

We agree that Sdk has modest effects on SCAR and Pyd accumulation in clones and we have now clarified in the manuscript the technical issues which would systematically under-detect the effects in fixed tissue (page 8). Specifically in live wildtype eyes, WRC and Pyd levels change during expansion and contraction. In fixed tissue it is not possible to classify the mechanical behavior of the contacts and relate molecular to mechanical dynamics. It is only possible to obtain averaged intensities across a population of contacts, an approach that is certain to underestimate the changes in protein levels as a function of expansion or contraction.

Another factor to consider with the fixed tissue results is that the magnitude of the effect is only one dimension of the interaction. That is, Sdk has a noteworthy effect on the timing of Pyd accumulation. In wildtype, Pyd accumulates during expansion and peaks during early contraction. However, this accumulation is significantly delayed in *sdk* mutants. Thus, although Sdk has modest effects on overall Pyd levels in fixed tissue, it strongly affects the timing of Pyd accumulation in live tissue. This result implies that Sdk controls the early phase of Pyd recruitment to vertices and that other mechanisms target Pyd to vertices during contraction as tension builds up. Given both factors, the ability to detect a statistically significant reduction in SCAR and Pyd levels in *sdk* mutant clones is quite striking, a point we clarify for the reader (page 8).

On top of the technical challenges of detecting changes in fixed tissue, the model we have developed incorporates the moderate effects we observed. That is, the model is that the interaction of Sdk with the WRC and Pyd provides robustness to the system. This robustness is conferred by pulsing of protrusive and contractile networks that we propose rebalances forces in the tissue. The loss of this rebalancing act therefore decreases robustness and leads to an increase in the frequency of remodeling errors. Thus, our results are consistent with the observations that *sdk* mutant adult flies are homozygous-viable with tissue abnormalities, while *pyd* mutants can survive to adulthood at low frequency, also with tissue abnormalities, consistent with more nuanced roles for these genes in epithelial remodeling, although with a more crucial requirement for *pyd*.

While loss of ‘robustness’ might not produce dramatic phenotypes, it is still deleterious and therefore obviously plays a key regulatory role. Regarding the WRC, the reduced protrusive actin dynamics in *sdk* mutants tells us that the engagement of the WRC is severely impacted, particularly with respect to pulsing. Our biochemical evidence that SCAR binds specifically to the Sdk C-terminal motif and the effect of overexpression of HA::Sdk on SCAR at vertices argue that a direct interaction between Sdk and the WRC *in vivo* mediates this effect. The fact that other mechanisms can partially compensate for the loss of the Sdk-SCAR interaction does not diminish its’ importance. Text changes in the manuscript have attempted to clarify this for the reader.

A point-by-point response to Reviewer's comments:

Reviewer 1

1-2. *Here, SCAR was shown to be pulled down by a fusion protein of the Sidekick cytoplasmic tail. In an earlier paper by the authors, Polychaetoid was shown to bind a fusion protein of the Sidekick cytoplasmic tail. Both interactions were lost when a terminal sequence was deleted from the Sidekick cytoplasmic tail, suggesting they bind to the same site. To confirm that they bind competitively to the same site, and to exclude that the site's deletion has non-specific effects on the fusion protein of the Sidekick cytoplasmic tail, the authors could test if the SCAR pull down by the Sidekick cytoplasmic tail is reduced by additional inclusion of a protein fusion of the Polychaetoid PDZ domains, shown by the authors to also bind the Sidekick cytoplasmic tail. Additional evidence for the competitive binding model could also come from testing Sidekick-WRC colocalization in a Polychaetoid mutant, or Sidekick-Polychaetoid colocalization in a WRC mutant.*

As discussed above, we addressed the issue of competitive binding genetically. We found a moderate and statistically significant increase in SCAR levels at vertices in clones of *pyd*^{ex147} null mutant cells compared to adjacent wildtype cells. This result further supports the idea that Pyd and the WRC bind to the same site in the Sdk protein.

3. *The authors "found that deleting the C-terminal motif from HA::Sdk almost abolished its strong dominant negative effects (Fig. 4H)" and used this data as an argument that Sdk functions with the WRC. However, the authors argue elsewhere that the WRC and Polychaetoid bind to the same site of Sdk. Thus, more caution should be taken in interpreting the results of deleting the C-terminal motif from HA::Sdk.*

Indeed, the HA::Sdk overexpression phenotype could arise from misregulation of both the WRC and Pyd. We now interpret the result more cautiously (page 9).

4. *In Fig 3B-F, showing additional single channel images is important for comparing the protein distributions. In particular, it is recommended to additionally show Sdk-GFP as single channel images.*

As suggested by the reviewer, we now show Sdk-GFP as single channel images for a better comparison with pMyoII (Fig. 3B) and SCAR (Fig. 3C-E) in the zoomed in panels.

5. *In Fig S2C-D, the phospho-myosin staining is not convincing-the described "apicomedial pool" is not obvious in the control, nor is its loss with the Rho kinase inhibitor. This issue might be due to non-specific/variable staining (much of the signal seems to be cytoplasmic since strong nuclear exclusion is apparent). I believe the change in the Sdk-GFP distribution can still be interpreted if the phospho-myosin staining was left out.*

As suggested by the reviewer, we removed the phospho-myosin staining channels from Fig. S2 and edited the text accordingly. We agree that we can still conclude from the data presented that actin assembly and actomyosin contractility concentrate Sdk at vertices.

Reviewer #2:

1. *Regarding the Intro: Some citations need reconsideration. For example, on Page 3 paragraph 2: Sentence 2 - Cagan and Carthew citations are correct, but the Galy, Del Signore and Zallen citations do not relate to this sentence. Sentence 3 - Bao and Cagan, 2005 is an odd citation choice here. Final few sentences - if all info discussed relates to Del Signore, perhaps insert this citation earlier?*

We moved the Galy, Zallen and Del Signore citations to the next sentence, which describes the role of mechanical forces in eye development. We included instead a new citation of a timely review on eye patterning and morphogenesis (Johnson, 2021) (page 3). We also removed the Bao and Cagan citation and added a new citation of a more relevant paper (Blackie et al. 2020) (page 3).

2. Regarding Figure 1 and associated text/supplementary data:
Broad comments on Figure 1:
1A - key could be more intuitive: a) needs more space to separate the words '2{degree sign}' 'doomed' and 'bristle' (I first read this as one label and wondered why you thought the bristles were doomed!). b) if shapes of cells in the key were taken directly from the ommatidia tracings, would be more intuitive (all shapes used are similar but not quite the same).

In the key to Fig. 1A, we spaced out the words and changed the shape of the cells to correspond to the traced cells in the ommatidium at 26h APF, as suggested.

1D - image should be rotated ~5{degree sign} clockwise to align along the dorsal-ventral axis of the eye.

We rotated panel 1D by 5 degrees as suggested.

1I,J,K,M,N .... Please make these panel numbers easier to see!

We placed the labels over a black background to make them more visible as suggested.

1O - I'd prefer to see an image of Sdk localization that encompasses a larger eye region. The central blue and green dots placed on the 2{degree sign} and 3{degree sign} cell can easily be mistaken for sdk-GFP puncta! Your figure legend states this tissue is 42 h APF, but in the manuscript states 36 h APF.

We replace the zoomed images with an image of an entire ommatidium and enlarged the circles that label the 2° and 3° lattice cells to make them easier to see. We also corrected the figure legend to say 36h APF.

Page 5 - the authors state that the cellular rosette phenotype is one 'that arises from defects in cell intercalation' but other defects can cause this too (eg. compromised cell survival, defects in primaries). Indeed you observe and report changes in cell survival and shape defects (which may

or may not be consequent to cell position). My objection here can be resolved by stating: '... cellular rosettes, a phenotype that can arise from defects in cell intercalation'.

We agree and edited the sentence as suggested.

Page 5 - from paragraph 2 - the authors jump from discussion of intercalation (previous paragraph) to comparison of the 'pumping' dynamics of LCs that occurs after intercalation. Please make it clear to readers that these events are temporally distinct, and that the live-imaging data relates to the later events.

We now include a sentence in page 5 to make this transition and state that "After cell intercalation, LC-LC contacts expand and contract dynamically, and new contacts form between LCs immediately after pruning."

As always, your live imaging is very good. But I have problems with the comparison of I and J:
a) why did you chose to compare behavior of LCs located horizontally (with respect to dorsal and ventral ommatidia) in wild type tissue, with LCs located obliquely in sdk[mut]? Are the dimensions and dynamics of cells in these different positions truly identical (in wild type)? There are (usually) fewer cells in oblique positions.
b) Also, in the sdk[mut], but not wt, one cell dies. You comment on re-establishment of the new LC-LC contact in the text - why this re-establishment is needed is hard to understand for non-eye experts (remedy: point out dying cell). But importantly, please provide wt data of a similar new LC-LC contact being established so that readers can see the comparison.
c) Since the central LC is dying in the sdk[mut] tissue, which pulsing membrane are readers to compare?
d) We also have a dying cell in K but comparisons between the green LC and unmarked LC to its right can be made. If this is your intention, perhaps indicate this with an arrow head?

Panel I shows the pulsing behavior of the LC-LC contacts in a wildtype eye, while J and K show both the pulsing behavior and other defects including loss of cells and cell contacts in *sdk* mutant eyes and eyes expressing HA::Sdk, respectively. Although we did not quantify pulsing in oblique contacts, we chose to present the image shown in panel J and the accompanying Movie 1 because it shows four major defects in *sdk* mutant eyes including intercalation defects, decreased amplitude of contact pulsing, separation of LC-LC contacts, and loss of LCs. The reviewer reasonably asks for examples of pulsing in *sdk* and *UAS-HA::Sdk* and pruning in wildtype. We replaced the wildtype eye panel in Movie 1 with a panel that shows normal pruning for a direct comparison with the aberrant pruning in experimental eyes demarcated with white rectangles. We also demarcated with yellow rectangles normal pulsing of LC-LC contacts in wildtype and aberrant pulsing in experimental eyes. We only quantified pulsing along horizontal edges of LCs in all four genotypes and now note it in the legend and method section. We edited the text to make it clearer that we see both a reduced pulse amplitude and separations of LC-LC contacts caused by improper pruning, which are two different aspects of the same phenotype (page 5).

e) Please indicate (in the text or figure legend) the age of the retinas at the start of live imaging. If 36 h APF, then data presented in panel O matches well. If earlier, please comment on whether Sdk localizes in the same way earlier.

We started imaging at 26-28hAPF and now note it in the legend. HA::Sdk localization in O is from a 36h APF eye and we note that HA::Sdk localizes in the same way at earlier stages (page 30).

Regarding Figure 2 and others:

Several superimposed white boxes (eg. Fig 2, lower panels of A, B and C; Fig 3A, B, etc.) are not easy to see (the borders of these are inconsistent - perhaps something odd happened when the pdf of the manuscript was built?).

The boxes indicated look clear to us in the current built-up version of the manuscript.

Regarding Figure 3 and associated text/supplementary data:

It's not clear why data is presented from inconsistent ages APF - Utr, Abi at 24 h, p-Sqh at 32 h. Presentation of Scar localization at 26, 32 and 40 h does make sense as this map changes in the localization of Sdk (great data!). Since in the text, you state that 'pumping' occurs from 24-28 h APF, analyses of p-Sqh within this window would make more sense. Analyses of Utr, Abi and p-Sqh at 26 h APF would more logically fit with the Sdk/SCAR data presented.

We replaced the Utr panel at 24h APF in Fig. 3A with a new panel at 28h APF as requested. We attempted to replace the Abi panel at 24h. However, at later stages there is an increase in mCherry aggregates at the apical part of the epithelium, which can obscure some of the Abi signal at LC-LC contacts. We therefore kept the original Abi panel. This panel is from a time-lapse movie, and we confirmed that the LC-LC contacts are pulsing at the stage shown.

Regarding Figure 4 and associated text/supplementary data:

I found your comment that 'HA::Sdk overexpression promotes dispersion and degradation of WRC subunits from the cell surface' hard to follow. Why the suggestion of degradation? Surely, since HA-Sdk mis-localizes to LC-primary cell boundaries, SCAR would be mislocalized there too? But do you observe this? I can't see this consistently in the data presented. Replacing 'cell surface' to 'cell boundary' or 'cell contact' would be more precise.

Indeed, we do not provide evidence to suggest that HA::Sdk promotes degradation of SCAR. Our data is more consistent with the idea that HA::Sdk promotes dispersion of the WRC. We edited the text accordingly.

Did you mean to include the boxed regions of panels A and C and higher resolution (larger?) That would be helpful to readers.

We included slightly larger panels for the boxed regions in panels A, C and D to help the reader appreciate the differences.

However, the GST pulldown data is more convincing. I would have liked to see more GST-Sdk

present in the Coomassie stain. As a matter of interest, what are the 2 lower bands below SCAR on the WB?

The two lower bands must be degradation product of the full-length GST-Sdk and GST-Sdk$^{\Delta CT}$ proteins. The Coomassie stain is from a pilot experiment of a small 2 ml culture where we only pulled down a relatively small amount of the GST proteins.

I would like to see - perhaps as a supplemental image - an image of SCAR localization in GMA>HA::Sdk-deltaCT.

We included an image of GMR>HA::Sdk-deltaCT in Fig. 4 (now panel 4G).

Given the weak changes in SCAR localization in sdk mutant LCs, please soften the language to make clear that Sdk promotes SCAR localization but is partially redundant.

We originally discussed the implication of this observation in the Discussion. We now also state in the Results that the observation that SCAR levels are not lost but just reduced in *sdk* mutant cells implies that other proteins in addition to Sdk localize the WRC to cell contacts (page 8).

What is the age APF of the data presented in Figure 4A, C, E, F? E and F look to be around 36-40 h APF which, according to Fig 3E is when less membrane-associated SCAR is observed. Would the effects of GMR>Sdk not be more convincing at 28 h APF?

A and C are at 26h APF during pruning while E-G are at 30h APF during late pruning to completion of pruning. At 40h APF LC-LC contacts are relatively stable, while at earlier stages during pulsing a subset of the contacts are “pinched” during contraction as seen in E and F. During the time interval shown in E-F, SCAR localizes to vertices and LC-LC contacts that are pinched implying that they are pulsing.

This is probably picky (!): Fig 4B - there's a disconnect between your use of tAJs/bAJs in this panel and 'vertices' in the text. Perhaps this could be solved by, somewhere earlier in the manuscript, clarifying how you are using these terms.

For consistency we changed the label from tAJs/bAJs to vertex/LC-LC contacts in 4B.

Regarding Figure 5 and associated text/supplementary data:

These data are gorgeous.

I'm confused about panels F-J: it's not completely clear whether these analyses are of Sdk at vertices only or along the entire LC-LC contact. Can you make this clear on the panels or in the figure legend? I assume that for panels C-D Sdk along the entire LC-LC contact was quantified? If this is incorrect, please make clearer.

The entire analysis in Fig. 5 was done on Sdk levels at vertices. We now edited the labels to the panels in Fig. 5 to indicate that.

Regarding Figure 6 and associated text/supplementary data:

Comment: GMR>RacN17 is very heavy handed, in that adherens junctions fail to form correctly and are poorly distributed in this genotype. This could account for or contribute to lack of localization of sdk-GFP in this genotype. You also state that expression of MLCK-ct , which increases tension, did not modify Sdk localization (did I misunderstand this phrase in your text as I see a difference in Fig 6?).

Indeed, Rac.N17 reduces overall F-actin levels and thereby tension that is also required for the stability of the adherens junctions as we originally noted in the Results. To increase the stability of the adherens junctions and maintain epithelial integrity and ommatidia structure in these experiments, we aged larvae at 18°C and then shifted pupae to 25°C to increase transgene expression prior to imaging (page 19).
We show that MLCK.ct disrupts the dynamic fluctuations of Sdk levels during contact contraction and expansion (Fig. 6D compared to wildtype in E-E' and quantification in F). We now indicate that the analysis in D corresponds to MLCK.ct image data in C in Fig. 6 and clarified it in the text (page 10).

Comment on Figure S2 - I wonder if the strange clumping of Sdk in B indicates (direct or indirect) cis-interactions between Sdk?

The accumulation of Sdk in string-like structures in response to Latrunculin A treatment in Fig. S2 may certainly arise from enhanced cis interactions between Sdk molecules, from enhanced trans interactions, or both. Regardless of the actual mechanism, actin dynamics appear to inhibit the formation and spreading of these Sdk clusters.

Regarding Figure 7 and associated text/supplementary data:

Again, beautiful live imaging data ....

You quantify the changes in Pyd vertex localization in response to loss of Sdk in comparison to Pyd at the center of an LC-LC boundary. Could you clarify that this was because localization of Pyd along LC-LC boundaries was unaffected by Sdk? The effect looks very mild, but stronger at tAJs/bAJs between LCs rather than at LC-primary vertices. Do your analyses support this? If yes, then would you agree that there is an additional mechanism of recruitment of Pyd to LC-primary junctions that's redundant with Sdk? Stating this ahead of your comment that '... Sdk controls the timing of Pyd recruitment ...' would not undermine your conclusions.

We reexamined the data in response to this comment. We found a small redistribution of Pyd from vertices to LC-LC contacts in *sdk* mutant clones as the Reviewer noticed from the image data. We therefore normalized Pyd levels at vertices to Pyd levels at LC-1° contacts, which do not change, to obtain cleaner results and updated the panel (Fig. 7J). We added a sentence that states that the results further suggest that additional mechanisms target Pyd to cell contacts aside from Sdk (page 12).

Please include 'APF' after '26 h' (page 12, line 2). I haven't checked whether this is needed elsewhere in the manuscript.

We added APF in page 12 and in a few other locations in the manuscript.

January 25, 2022

RE: JCB Manuscript #202107035R

Prof. Victor Hatini
TUfts University School of Medicine
Development, Molecular & Chemical Biology (DMCB)
150 Harrison Avenue
Boston 02111

Dear Prof. Hatini:

Thank you for submitting your revised manuscript entitled "Sidekick dynamically rebalances contractile and protrusive forces to control tissue morphogenesis". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, unless you disagree with the suggestion please replace Figure 1I with better data as suggested by Reviewer #2.

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

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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 (either in the figure legend itself or 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."

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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 in the text for readers who may not have access to referenced manuscripts.

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

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Kenneth Yamada, MD, PhD
Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors effectively addressed my past concerns. Overall, the revised manuscript is well-written and the data are clear. The authors' discovery of Sidekick's direct involvement in coordinating the regulation of contractile and expansive actin networks during epithelial cell-cell contact remodeling is an important advance for the field.

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

I must begin by thanking the authors for nicely addressing each of my original concerns/comments. They have done an excellent job in revising and bolstering the manuscript, with the addition of Figure 8. They explain too the difficulties in acquiring data that would solidify their model of competitive binding of Pyd and WRC to Sdk. They have taken care to explain the caveats of their model, and data. This manuscript is ready for publication.

However I do still have comments about Figure 1I, which provides excerpts of live-imaging documenting cyclical LC-LC expansion and contraction. In my opinion, the wild type movie data provided in Movie S1 is better than that presented in Figure 1I and I'm surprised that Figure 1I was not replaced with data from Movie S1 in response to my initial criticism on this figure (first submission review). I leave it up to the JCB editor to decide on whether requesting that these data be changed is reasonable. Readers who navigate to Movie S1 will of course see the nicer data, but in my opinion, changing these data will avoid a criticism from readers. To explain: on pouring over the resubmitted Figure 1, I again had the same criticism as before: we are asked to compare LC:LC behavior in wt (panel I), sdk[mutant] (panel J) and sdk[+] (panel K) retinas. However in the latter two genotypes, the LC:LC boundary highlighted is next to a dying cell, but not in the wt data. Surely the apoptosis and associated removal of a cell from the epithelium will introduce forces into the tissue that could modify the LC:LC boundary behavior, and hence this is not the appropriate control data? Replacing panel 1I with stills from the wt data in Movie S1 (outlined in white in the movie) completely eliminates my criticism.