Rap1 regulates apical contractility to allow embryonic morphogenesis without tissue disruption and acts in part via Canoe-independent mechanisms

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Review Timeline:

Submission Date:	2022-05-18
Editorial Decision:	2022-06-23
Revision Received:	2022-10-06
Accepted:	2022-10-19

Editor-in-Chief: Matthew Welch

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.)

RE: Manuscript #E22-05-0176
TITLE: Rap1 uses Canoe-dependent and Canoe-independent mechanisms to regulate apical contractility and allow embryonic morphogenesis without tissue disruption

Dear Mark,

Thank you for sending us your MS for consideration at MBoC. It has now been seen by three expert reviewers, whose comments follow below.

As you can see, all the reviewers are supportive. But they also have suggestions to strengthen the paper. Some of these involve reinforcing the data with additional experiments, but also thoughts on how its presentation could be streamlined so that the message is as clear as possible.

Accordingly, let me ask you and your colleagues to revise your MS in light of these comments.

We hope to see that revised manuscript soon.

Best wishes,
Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Peifer,

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

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

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

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

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

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

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office

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Reviewer #1 (Remarks to the Author):

REMARKS TO THE AUTHORS

This manuscript addresses the role of the small RasGTPase Rap1 and its regulation during Drosophila embryonic morphogenesis. Previous studies have shown that Rap1 uses its effector Canoe to regulate the connection between junctions and the cytoskeleton during cellularization and mesoderm invagination. The authors now explored if Rap1 regulates intercellular adhesion and cytoskeleton organization during later stages of embryogenesis. In particular, the manuscript tests the hypothesis that Rap1 has other relevant effectors during Drosophila embryogenesis and examines if Dizzy is the main GEF regulating Rap1 function.

The introduction is very well written, making clear the implication of Rap1 function across very diverse biological contexts and the known Rap1 regulators and effectors from flies to mammals. The study is mostly based on the phenotypic comparison between rap1, cno and dizzy loss of function to determine which embryonic phenotypes are caused by the Dizzy-Rap1-cCno pathway or instead reflect independent functions of Rap1. The images are very clear and easy to follow with their detailed description, and I commend the authors for presenting a detailed analysis of how the phenotypes develop during different stages of embryogenesis.

These phenotypic analyses suggest that Rap1 acts via Canoe on the organization of junctional planar polarity, but it also has additional phenotypes. In particular, the authors observe fragmentation of apical junctions and Baz distribution and also a dramatic unbalance of apical contractility, which leads to massive constriction of cells surrounding mitotic domains. There is careful characterization of how these phenotypes develop from stage 5 to 13 of embryogenesis revealing that Rap1 roles go beyond its functions with Canoe and eventually leads to massive loss of epidermal integrity.
The authors then asked if Dizzy, which was previously described as an important regulator of Rap1-Cno pathway would also be the prevalent GEF involved in Rap1 regulation during embryonic morphogenesis during later stages. With that end, a new RNAi line was generated and used for phenotypic comparison with previous genetic perturbation with Rap1/Cno. They show Dizzy mutants reproduce a variety of Cno phenotypes, including defects in germband elongation and the formation of epithelial gaps, but do not recapitulate all phenotypes including the planar polarization defects during stage 7 of embryogenesis. More importantly, junctional fragmentation and severe defects in apical constriction is not an outcome of Dizzy RNAi, for which epithelial integrity is more generally maintained than in Rap1 mutants. In fact, the defects produced by Dizzy RNAi are more similar to those of canoe mutants lacking the Rap1 binding domain. Thus, it is proposed that Dzy is the main GEF regulating Rap1 interaction with the RAS-associated domain of the effector Cno, but there must be other GEF that is responsible Rap1 activation towards different Rap1 effectors that are relevant to balance apical contractility and maintain epithelial integrity. Overall, the findings that Rap1 loss of function leads to new Canoe/Dizzy independent phenotypes and produces unbalanced apical contractility are novel and can represent an important step towards the understanding of how Rap1 small GTPases regulate epithelial architecture.

Specific points:

1. The manuscript does a great job with the very detailed analysis of the effect of Rap1 on the localization and planar polarity of junctional proteins during the stages of germband extension, but does not describe what is the exact impact of Rap1 depletion on the process of germband extension at the whole animal level. This is however done for dzy RNAi in Fig. 7, and so it would be important to show data for Rap1 to allow comparison with the effects of dizzy loss of function at the whole animal level.

2. Figure 3Q and 3S: The manuscript states that in Rap1 RNAi, dorsal mitotic cells fail to resume columnar architecture. This assumes that the cells in Figure 3S (brackets) are indeed post-mitotic cells, but alternatively there could be problems with mitotic progression delaying the timing of the mitotic domains. A staining with any mitotic marker (e.g. phospho-histone3) would be useful to clarify if cells failed to recover shape and junctional organization after division of if they were still undergoing cell division.

3. The last paragraph of page 8 starts " The alterations in AJ protein localization observed at stages 7 and 8 continued during stage 9." and describes data from figure 4, that is partly redundant with the previous paragraph since it already describes stage 9 (Fig.3O to 3V). For instance, Fig. 4B describes the finding that all dorsal cell remain rounded, as previously stated for the description of severe embryos ("dorsal ectodermal cells fail to resume columnar architecture after their division at stage 8 (Fig. 3Q, R vs S, brackets"). Reorganization of the description of stage 9 between these paragraphs is needed to make clear that Fig.4 is a more detailed analysis of the severe phenotypes of stage 9. I assumed this was the case even if the title of Fig. 4 legend is "At stages 8 and 9, while core AJ proteins remain at cell junctions, defects in balanced contractility and junctional Baz localization continue or intensify." This title could be changed accordingly.

4 - Fig. 6 - The authors created a new RNAi line to evaluate Dizzy function during embryogenesis, and due the lack of an available antibody they evaluated depletion efficiency by comparison with mat/zygotic mutants. In fig. 6P there is a quantification of the analysis of cuticle phenotypes that is particularly useful by bringing in a direct comparison to the maternal zygotic nulls. There is a clear trend in the graph, but the data would be more compelling if the quantification indicated sample size and a statistical test evaluating differences in severity.

5 - In pag. 15, the manuscript indicates that for Dizzy RNAi dorsal ectodermal cells are also hyperconstricting next to mitotic domains "Dorsal ectodermal cells were hyper-constricted along the AP axis (Fig. 9L vs M, N, cyan brackets), perhaps due to reduced pulling from mitotic neighbors. ". This phenotype can relate to the unbalanced apical constriction observed for Rap1 RNAi, but this is not discussed in the manuscript. In fact, table S1 indicates that there are no widespread defects in unbalanced apical contractility for Dizzy RNAi. I think it is worth to clarify if the data suggests that regulation of apical contractility requires, at least partly, Dizzy function, and explain if difference to Rap1 is related to the strength of phenotype or the stage/location when this effect is apparent.

Minor points:

- Title suggestion: The authors may consider revising the title to focus on the aspects of the study that are particularly new, such as the identification of phenotypes that highlight Rap1 functions that are independent of Dizzy/Canoe. Also, the current title could indicate that the function of Rap1 in relation of apical contractility also relied on Canoe-dependent mechanisms. This is not as evident in the manuscript since it reports that there are no widespread changes of apical contractility in cno null mutants. So I wondered if the authors should reconsider removing the mention to Canoe-dependent mechanisms in the title.

- One suggestion for panels describing different phenotypic strengths for rap1 would be labelling the rap1 mutant panels with severe vs mild, this would quickly convey meaning when observing the figures with the multiple rap1 RNAi panels with the same stainings and stage. (e.g. Figure 5B and 5C).

- Figure 3P - Rap1 label is missing on the top right corner

- Fig 6K-6P are described in the text after the description of Fig 6Q-S. I suggest reorganizing the panels in the same order of the text.

- The authors may consider using the GFP-tagged Dzy driven by its endogenous promotor that they used in Bonello et al., 2018 as a tool to test the efficiency of depletion produced by the new RNAi line. This is not essential at this stage, since depletion seems efficient based on phenotypic analysis. I leave this option to strengthen the data in case there is sufficient time during revision.

- Fig.8 legend: Page 35 - "cells between the domains apically constrict as their neighbors reduce contractility (cyan arrows)" - I suggest modifying to make clear that neighbors refer to mitotic cells (mitotic neighbors) and that contractility is reduced at the apical level.

Reviewer #2 (Remarks to the Author):

In this manuscript, Perez-Vale and colleagues provide new data on the epistatic relationship between the GTPase Rap1 and the Afadin protein Canoe and their role in morphogenesis and epithelial stability in Drosophila embryo. Based on extensive previous work from the same group (who first characterised Canoe in flies), they complemented former studies by characterising and comparing thoroughly the embryonic phenotype of Rap1 depletion, Canoe depletion and the KD of an upstream GEF Dizzy. Based on careful and exhaustive phenotypic characterisation compared with some quantifications of planar polarity, they outlined several phenotypic features specific of Rap1 which are not visible in Canoe mutant, suggesting that Rap1 has other functions for epithelial stability and morphogenesis independent of Canoe. Similary, they found that Dizzy depletion produces milder phenotype (hence suggesting that this is not the only Rap1 GEF acting during early embryogenesis), which yet recapitulates most of the features previously observed with a Canoe mutant lacking the Rap1 interacting domain (CnoDRA Perez-Vale 2021). By essence, this work is mostly a classic genetic epistatic work based on solid and careful phenotype description. The conclusion are clear and interesting although admittedly the work does not necessarily provide much inside about the cause of phenotypes and mechanistic understanding of epithelial stability. Still, since the main conclusion of the work are related to this epistatic relationship, I believe some additional would be required to be fully conclusive. Moreover, I believe it would be important to really discuss the novel insights compared to numerous work from the same lab that already compared Cno and Rap1 mutant phenotypes (albeit at slightly earlier stages, but there was already some characterisation at stage 7, see Choi et al 2013 Mboc). I include below some suggestions along these lines :

1. All the conclusions of this work are based on single dsRNA lines both for Rap1 and dzy. While the phenotypes recapitulate features previously observed with maternal/zygotic null, it would be really important to be sure there is no off-target effects (since the conclusion are purely genetic epistatic relationship, it seems rather essential). Along these lines, could the authors:
- Check whether some of the specific features of the Rap1 KD phenotype (epithelial rupture, and/or Baz dispersion and/or heterogeneity of cell size) are recapitulated either with another RNAi or best, with the M/Z mutant the authors previously used ? (Choi et al 2013). I suspect one of the reason for not using the M/Z mutant is the ambiguity for the paternal allele which can be either WT or mutant, but observing qualitatively the same phenotype in the "strongly" impaired Rap1M/putativeZ embryo would already be an important validation.
- Similarly, since the authors cannot provide evidence for Dizzy depletion with their RNAi line, and apparently cannot use the maternal zygotic mutant, can they show that the phenotype is indeed rescued by providing a transgene not targeted by the dsRNA ? I suspect this will be difficult since the targeted region is in a codon, but there might be way to solve this using codon degeneration and generating a dizzy transgene poorly targeted by their construct ? (either by transgenesis or just mRNA injection).

These two controls seem quite essential as the phenotype observed are not necessarily that specific of the targeted genes (similar elements can be observed with partial depletion of polarity of adhesive components as previously documented by the authors).

2. As discussed above, it was not clear to which extend the speficities of Rap1 phenotypes were totally novel. For instance, the heterogeneity of cell apical area was already described in Choi et al 2013 (albeit at earlier developmental stage). Similarly the authors already showed part of the phenotype at stage 7. In a way, it was not necessarly obvious how the previous data were not sufficient to reach the same conclusion about the existence of other Rap1 targets. It might be useful to state more clearly which observations are completely novel (specially based on the numerous previous work from the same laboratory) either in the discussion or in the introduction.

3. Two important features of the Rap1 phenotype are related to heterogeneity of cell size (which the authors propose to be driven by heterogeneity of tension) at stage 7 and near dividing cells, and early disruption of Bazooka. As mentioned above, at this stage it is hard to know to which extend morphogenesis defects are mostly driven by disruption of adhesion or misregulation of tension (or both). It will probably hard to sort these contributions since there is a lot of mutual regulation, but it might be good to document at least the distribution and levels of MyoII in these genotypes (comparing Rap1 KD, Canoe mutant and Dizzy KD). This will help to see whether there is a global increase/decrease or just an increases heterogeneity of MyoII between cells. This information might have already been published by the group, and in that case it would be important to include this information in the discussion.

4. If tissue rupture is indeed preferentially formed in regions of high tension, would it be possible to partially rescue this phenotype by a mild reduction of tension ? (by reducing Rho and/or ROCK activity either genetically or using low concentration drug injection). Admittedly, this might be a difficult experiment to perform, if the point of the article is purely epistatic it might not be essential for this publication, but it would for sure add some mechanistic explanation. Maybe there is already published data along this line and it might be interesting to discuss it.

Other minor points :
- So far the authors provide information about canoe planar polarised distribution upon depletion of Rap1 and Dizzy. What about apico-basal distribution and global levels ? Based on previous results from the group, I assumed that Canoe cortical recruitment was clearly reduced upon Rap1 depletion. Is that the case here as well (specially compared to dzy RNAi and CnoDRA) ?
- Figure 9 : could the authors show the armadillo channel separately to visualise the planar polarity ?
- There are some striking similarities between the phenotype of aPKC mutant and Rap1 depletion (see notably the higher variability of cell apical area already described in Choi et al 2013). Along the point in the discussion about the effect of partial depletion of aPKC (results for Eurico Moraes de Sa lab), this might be an interesting element to add.

Reviewer #3 (Remarks to the Author):

In this manuscript, Perez-Vale, Yow, and the team investigated how Canoe/Afadin, which is associated with adherens junction, a small GTPase Rap1, and a GEF regulators Dizzy, influence each together during early Drosophila embryogenesis. To this end, the authors compared different phenotypes, including planar polarity at the cell-cell junction, cell shape, and tissue integrity at different embryogenesis, between wildtype and mutants, including Rap1 RNAi, cno mutant, and dzy RNAi. Depending on the similarity/difference of phenotypes in each fly line, the authors characterize whether Dizzy regulates Canoe via Rap1 or whether other GEFs play roles in regulating Canoe through Rap1. The authors concluded that this is context-dependent.
Overall, this study is addressing important but challenging questions. Most of the data and interpretations are solid and intriguing (except a few I highlight below), and I would support publication in MBoC provided some of the concerns I list below can be addressed.

Major comments:

#1. I feel that the first half of the result section, which is characterizing the phenotype of Rap1 RNAi (Figs. 1-5), is hard to follow. Similar to what the authors did at the begging of the second half of the results using dzy RNAi (Figs. 6-10), it would be helpful if the authors state at the beginning of this result section (page 5 middle) that:
- The first goal is to determine which Rap1's roles during embryogenesis require the Rap1 effector Cno, and which ones don't.
- To this end, the authors compared different phenotypes, including planar polarity at the cell-cell junction, cell shape, and tissue integrity at different embryogenesis, between wildtype and mutants, including Rap1 RNAi, cno mutant.
- The authors envisioned two scenarios. The first group of Rap1-dependent morphological characteristics is Cno dependent. In this case, Rap1 loss would mimic the loss of Cno. The second group is Cno independent.
- "explore the roles of Rap1 and its **regulators** during morphogenesis" (page 5 middle) sounds confusing. I believe this section is not to investigate the upstream of Rap1, i.e., regulators, but the downstream effects.
These lines could help the reader understand the structure and idea behind the lengthy result section (Figs. 1-5).

#2. The first section of the results "While Rap1 RNAi mimics the effect of Cno loss on AJ planar polarity..." is confusing.
- I suggest citing their own data, including the distribution of Cno, instead of simply citing the previous paper, in the first paragraph of page 6.
- I suggest adding Pyd images in Fig.1. It looks strange not to show the images but provide the quantifications in Fig. 2B.
- "Bazooka (fly Par3; Baz) is obviously planar polarized at this stage..." It would be helpful to specify Bazooka is more on DV borders than AP borders, instead of "planar polarized".
- It is not clear whether the 4-line-long segment starting with "In contrast, Cno loss enhances..." represents the results from this study or the findings from the other papers. Please cite the data or the references.
- What "Cno loss" means? Are you referring to cno mutant? Please specify.
- "by strongly reducing their accumulation on AP borders." How do you know this is only due to the reduction at AP borders? How about the accumulation at DV borders? Or the combination?
- "core AJ proteins remain localized to AJs in cno mutants." Which data shows this?
- "While Arm and Cno return to apical AJs..." Where do Arm and Cno return from?
- "places where both Arm and Pyd were also enriched (Fig. 1E", inset)." I suggest adding a rectangle representing the same ROI in E'. Moreover, a red arrow is missing in E'.
- When explaining the changings in planar polarity shown in Fig. 2A-D, the authors used the terms "elevating", "reversed", and "enhanced". I suggest explicitly explaining 1) "reversed" represents the localization change from AP>DV to DV>AP, and 2) "elevating" and "enhanced" represent the changes from AP>DP to AP>>DP.

#. "Together these data suggest Dzy function is... but is important to reinforce AJs under elevated tension, like TCJs and constricting AP borders. (page 13 bottom)". Please specify which data support the statement "Dzy function is important for constricting AP borders"?

#. I feel the statement "Dzy is required for ... Cno tension sensing" is a bit of a stretch. The authors show "Cno TCJ enrichment was strongly reduced after dzy RNAi (Fig. 8A vs B)". However, the reduction of Cno at TCJ does not simply mean the tension sensing via Cno is compromised. To make this claim, junctional tension needs to be measured (for instance by laser ablation). Alternatively, the authors can simply highlight the role of Dzy in Cno localization at TCJ.

Minor comments:

#. "horizonal borders" can be replaced with "DV borders".

#. I feel there are too many "dramatic", "striking", or "drastic" observations.

#. I do not see "fold inward (page 8 top)" in Fig. 3D.

#. I do not see "and AJ gaps at AP borders and TCJs (page 8 top)" in Fig. 3G. Adding arrows should be helpful.

#. It would be informative to quantify the gap in Rap1 RNAi (Fig. 3), similar to Fig. 8I.

#. "Fig. 8D, E, J, yellow arrows (page 13 middle)". There is no Fig. 8J.

Response to Reviews MBoC #E22-05-0176

We are very grateful to the reviewers for their generally supportive response to our initial manuscript and their very helpful feedback. The three reviewers had a diverse set of suggestions for strengthening the manuscript. Prompted by their suggestions, over the past several months we carried out multiple new experiments to strengthen the mechanistic insights, including most of those suggested by the Reviewers. We verified that the effects of *Rap1* RNAi are due to Rap1 on-target effects, by using an alternate approach to reduce function—a GDP-locked Rap1 dominant negative. We analyzed the effect of *Rap1* RNAi on germband extension, revealing it has an earlier and more severe effect than loss of Cno or dzy RNAi. We verified that *Rap1* RNAi does not prevent apical enrichment of AJs once gastrulation is underway, but that it reduces but does not eliminate junctional Arm and Cno localization. We added images documenting the effect of *Rap1* RNAi on Pyd planar polarity. We added new analysis of the effect of *Rap1* RNAi on myosin localization, and on junctional aPKC. We added numbers of embryos and did statistical tests on our comparisons of the effects of *dzy M/Z* mutants and our *dzy* RNAi approach, and provide experimental support for the idea that the *dzy* RNAi effects are also on-target. These data are included in new Figures 7, S1, and S2 and Revised Figures 1, 2, 4, 8, and 9 (note that all Figures were renumbered). We have also followed the Reviewers' excellent suggestions about revising the text to make our goals and approaches clearer to the reader, and by making additional alterations to our Figures. We have included a "Marked Up" copy of the text with significant changes marked in red. The revisions have substantially strengthened the manuscript and hope the Reviewers agree.

Reviewer #1 (Remarks to the Author):

REMARKS TO THE AUTHORS

This manuscript addresses the role of the small RasGTPase Rap1 and its regulation during Drosophila embryonic morphogenesis. Previous studies have shown that Rap1 uses its effector Canoe to regulate the connection between junctions and the cytoskeleton during cellularization and mesoderm invagination. The authors now explored if Rap1 regulates intercellular adhesion and cytoskeleton organization during later stages of embryogenesis. In particular, the manuscript tests the hypothesis that Rap1 has other relevant effectors during Drosophila embryogenesis and examines if Dizzy is the main GEF regulating Rap1 function.

The introduction is very well written, making clear the implication of Rap1 function across very diverse biological contexts and the known Rap1 regulators and effectors from flies to mammals. The study is mostly based on the phenotypic comparison between rap1, cno and dizzy loss of function to determine which embryonic phenotypes are caused by the Dizzy-Rap1-cCno pathway or instead reflect independent functions of Rap1. The images are very clear and easy to follow with their detailed description, and I commend the authors for presenting a detailed analysis of how the phenotypes develop during different stages of embryogenesis.

These phenotypic analyses suggest that Rap1 acts via Canoe on the organization of junctional planar polarity, but it also has additional phenotypes. In particular, the authors observe

fragmentation of apical junctions and Baz distribution and also a dramatic unbalance of apical contractility, which leads to massive constriction of cells surrounding mitotic domains. There is careful characterization of how these phenotypes develop from stage 5 to 13 of embryogenesis revealing that Rap1 roles go beyond its functions with Canoe and eventually leads to massive loss of epidermal integrity.
The authors then asked if Dizzy, which was previously described as an important regulator of Rap1-Cno pathway would also be the prevalent GEF involved in Rap1 regulation during embryonic morphogenesis during later stages. With that end, a new RNAi line was generated and used for phenotypic comparison with previous genetic perturbation with Rap1/Cno. They show Dizzy mutants reproduce a variety of Cno phenotypes, including defects in germband elongation and the formation of epithelial gaps, but do not recapitulate all phenotypes including the planar polarization defects during stage 7 of embryogenesis. More importantly, junctional fragmentation and severe defects in apical constriction is not an outcome of Dizzy RNAi, for which epithelial integrity is more generally maintained than in Rap1 mutants. In fact, the defects produced by Dizzy RNAi are more similar to those of canoe mutants lacking the Rap1 binding domain. Thus, it is proposed that Dzy is the main GEF regulating Rap1 interaction with the RAS-associated domain of the effector Cno, but there must be other GEF that is responsible Rap1 activation towards different Rap1 effectors that are relevant to balance apical contractility and maintain epithelial integrity. Overall, the findings that Rap1 loss of function leads to new Canoe/Dizzy independent phenotypes and produces unbalanced apical contractility are novel and can represent an important step towards the understanding of how Rap1 small GTPases regulate epithelial architecture.

We're grateful for your positive feedback about the work

Specific points:

1. The manuscript does a great job with the very detailed analysis of the effect of Rap1 on the localization and planar polarity of junctional proteins during the stages of germband extension, but does not describe what is the exact impact of Rap1 depletion on the process of germband extension at the whole animal level. This is however done for dzy RNAi in Fig. 7, and so it would be important to show data for Rap1 to allow comparison with the effects of dizzy loss of function at the whole animal level.

This was an excellent suggestion and yielded an interesting result. Rap1 depletion has a strong effect on germband extension, and this effect is more severe than that of Dzy knockdown in two ways. Dzy knockdown did not alter the earliest phase of germband extension (during stage 7), but only delayed things later (stages 8 and 9). In contrast, Rap1 depletion reduced germband extension from the beginning, and also more substantially impaired extension at later stages. This is consistent with another observed difference between depletion of Rap1 and loss/reduction of Cno and Dzy—Rap1 depletion often leads to a "twisted gastrulation phenotype", something we observe less frequently after Dzy or Cno knockdown. These data are now in revised Figure 2. These data reinforce the closer match between dzy RNAi and Cno loss,

which in contrast to Rap1 RNAi have similar effects during germband extension.

2. Figure 3Q and 3S: The manuscript states that in Rap1 RNAi, dorsal mitotic cells fail to resume columnar architecture. This assumes that the cells in Figure 3S (brackets) are indeed post-mitotic cells, but alternatively there could be problems with mitotic progression delaying the timing of the mitotic domains. A staining with any mitotic marker (e.g. phospho-histone3) would be useful to clarify if cells failed to recover shape and junctional organization after division of if they were still undergoing cell division.

This was an excellent suggestion. We stained Rap1 RNAi embryos with anti-phospho-histone3—an example of this data is included in revised Figure 3T. This confirmed that some but not all rounded up cells were post-mitotic. We have softened the text as follows. "In more severe embryos (9/23 scored), some ectodermal cells failed to resume columnar architecture after their divisions (Fig. 3Q, R vs S, brackets). We verified that some of these rounded up cells had completed mitosis by staining for the mitotic marker phosphohistone3, which only stained some of the rounded up cells (Fig. 3T, yellow vs cyan arrowheads)."

3. The last paragraph of page 8 starts " The alterations in AJ protein localization observed at stages 7 and 8 continued during stage 9." and describes data from figure 4, that is partly redundant with the previous paragraph since it already describes stage 9 (Fig.3O to 3V). For instance, Fig. 4B describes the finding that all dorsal cell remain rounded, as previously stated for the description of severe embryos ("dorsal ectodermal cells fail to resume columnar architecture after their division at stage 8 (Fig. 3Q, R vs S, brackets"). Reorganization of the description of stage 9 between these paragraphs is needed to make clear that Fig.4 is a more detailed analysis of the severe phenotypes of stage 9. I assumed this was the case even if the title of Fig. 4 legend is "At stages 8 and 9, while core AJ proteins remain at cell junctions, defects in balanced contractility and junctional Baz localization continue or intensify." This title could be changed accordingly.

This critique was embarrassingly right on target. We have now revised the text and Figure legend to clarify that in Figure 4 is focused on junctional protein localization, and removed the redundant description of changes in cell shape.

4 - Fig. 6 - The authors created a new RNAi line to evaluate Dizzy function during embryogenesis, and due the lack of an available antibody they evaluated depletion efficiency by comparison with mat/zygotic mutants. In fig. 6P there is a quantification of the analysis of cuticle phenotypes that is particularly useful by bringing in a direct comparison to the maternal zygotic nulls. There is a clear trend in the graph, but the data would be more compelling if the quantification indicated sample size and a statistical test evaluating differences in severity.

Excellent point. We have added the numbers of cuticles scored to the Figure (both ≥480), and, after consultation with a statistically knowledgeable colleague, used a Chi-square contingency test based on the number of embryos in each phenotypic category ($p<0.0001$). We then revised

the text as follows, to emphasize that both genotypes deviate substantially from wildtype, but that the dzy RNAi approach led to slightly weaker defects.

"Embryos from the dzy$^{\Delta 1}$ dzy RNAi cross exhibited a similar range of phenotypes (Fig. 6S), with nearly universal defects in head involution, and with many embryos having holes in the cuticle. The distribution of phenotypes was somewhat less severe in the progeny of the dzy RNAi cross than among dzy$^{\Delta 1}$ maternal/zygotic null mutants ($p<0.0001$, Chi-square test), but given that only 25% of the dzy$^{\Delta 1}$ dzy RNAi embryos would be predicted to also be zygotically dzy null mutant, the overlap in cuticle phenotype suggests we have achieved strong reduction of maternal and zygotic Dzy function in this subset of embryos, and thus our strategy provided a reagent for defining the role of Dzy during morphogenesis."

5 - In page 15, the manuscript indicates that for Dizzy RNAi dorsal ectodermal cells are also hyperconstricting next to mitotic domains "Dorsal ectodermal cells were hyper-constricted along the AP axis (Fig. 9L vs M, N, cyan brackets), perhaps due to reduced pulling from mitotic neighbors. ". This phenotype can relate to the unbalanced apical constriction observed for Rap1 RNAi, but this is not discussed in the manuscript. In fact, table S1 indicates that there are no widespread defects in unbalanced apical contractility for Dizzy RNAi. I think it is worth to clarify if the data suggests that regulation of apical contractility requires, at least partly, Dizzy function, and explain if difference to Rap1 is related to the strength of phenotype or the stage/location when this effect is apparent.

This was also an excellent point that needed clarification. Dzy knockdown, and from our earlier work, cnoΔRA or strong Cno knockdown, do lead to modest alterations in dorsal ectodermal apical cell area at stage 9. However, this is much less dramatic than the strongly unbalanced apical constriction see after Rap1 RNAi—these are seen earlier (stage 8) and become quite severe, leading to tissue infolding. We have revised this section to read: "Dorsal ectodermal cells were somewhat hyper-constricted along the AP axis (Fig. 9L vs M, N, cyan brackets). However, this contrasted with the earlier onset and much stronger hyper-constriction observed after Rap1 RNAi, leading to tissue infolding—instead, the dzy RNAi defect was more similar to what we observed in cnoΔRA mutants (Perez-Vale et al., 2021)." We also modified Table 1 to make this clearer.

Minor points:

- Title suggestion: The authors may consider revising the title to focus on the aspects of the study that are particularly new, such as the identification of phenotypes that highlight Rap1 functions that are independent of Dizzy/Canoe. Also, the current title could indicate that the function of Rap1 in relation of apical contractility also relied on Canoe-dependent mechanisms. This is not as evident in the manuscript since it reports that there are no widespread changes of apical contractility in cno null mutants. So I wondered if the authors should reconsider removing the mention to Canoe-dependent mechanisms in the title.

Good idea—we revised and rearranged the title to make these points more clearly

- One suggestion for panels describing different phenotypic strengths for rap1 would be labelling the rap1 mutant panels with severe vs mild, this would quickly convey meaning when observing the figures with the multiple rap1 RNAi panels with the same stainings and stage. (e.g. Figure 5B and 5C).

Good point—added as suggested

- Figure 3P - Rap1 label is missing on the top right corner

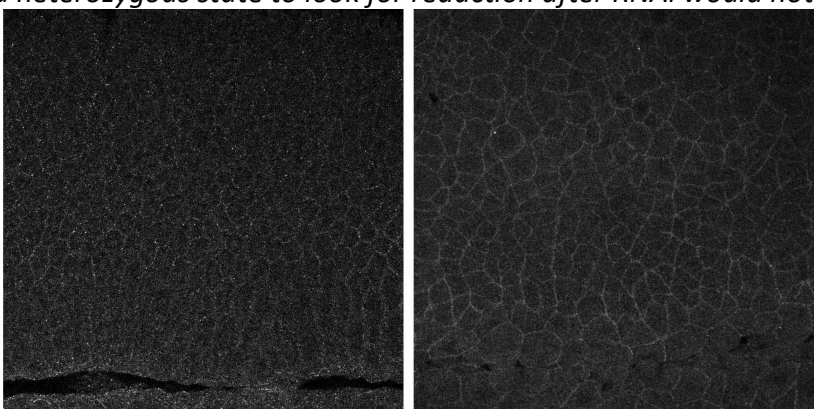
Fixed—thanks!

- Fig 6K-6P are described in the text after the description of Fig 6Q-S. I suggest reorganizing the panels in the same order of the text.

Good suggestion—altered as suggested

- The authors may consider using the GFP-tagged Dzy driven by its endogenous promotor that they used in Bonello et al., 2018 as a tool to test the efficiency of depletion produced by the new RNAi line. This is not essential at this stage, since depletion seems efficient based on phenotypic analysis. I leave this option to strengthen the data in case there is sufficient time during revision.

We put quite a bit of effort into this suggestion, which was definitely worth a try. Our efforts to visualize the GFP directly failed, and thus we used anti-GFP antibodies The end result is that the cortical signal from the Dzy-GFP line was pretty weak and variable, even in embryos maternally and zygotically homozygous (see the images below). Our colleagues in the McDonald lab in Kansas have had similar experiences in the tissues they study. This left us feeling that using it in a heterozygous state to look for reduction after RNAi would not provide a reliable answer.



- Fig.8 legend: Page 35 - "cells between the domains apically constrict as their neighbors reduce

contractility (cyan arrows)" - I suggest modifying to make clear that neighbors refer to mitotic cells (mitotic neighbors) and that contractility is reduced at the apical level.

Altered as suggested

Reviewer #2 (Remarks to the Author):

In this manuscript, Perez-Vale and colleagues provide new data on the epistatic relationship between the GTPase Rap1 and the Afadin protein Canoe and their role in morphogenesis and epithelial stability in Drosophila embryo. Based on extensive previous work from the same group (who first characterised Canoe in flies), they complemented former studies by characterising and comparing thoroughly the embryonic phenotype of Rap1 depletion, Canoe depletion and the KD of an upstream GEF Dizzy. Based on careful and exhaustive phenotypic characterisation compared with some quantifications of planar polarity, they outlined several phenotypic features specific of Rap1 which are not visible in Canoe mutant, suggesting that Rap1 has other functions for epithelial stability and morphogenesis independent of Canoe. Similary, they found that Dizzy depletion produces milder phenotype (hence suggesting that this is not the only Rap1 GEF acting during early embryogenesis), which yet recapitulates most of the features previously observed with a Canoe mutant lacking the Rap1 interacting domain (CnoDRA Perez-Vale 2021). By essence, this work is mostly a classic genetic epistatic work based on solid and careful phenotype description. The conclusion are clear and interesting although admittedly the work does not necessarily provide much inside about the cause of phenotypes and mechanistic understanding of epithelial stability. Still, since the main conclusion of the work are related to this epistatic relationship, I believe some additional would be required to be fully conclusive. Moreover, I believe it would be important to really discuss the novel insights compared to numerous work from the same lab that already compared Cno and Rap1 mutant phenotypes (albeit at slightly earlier stages, but there was already some characterisation at stage 7, see Choi et al 2013 Mboc). I include below some suggestions along these lines :

1. All the conclusions of this work are based on single dsRNA lines both for Rap1 and dzy. While the phenotypes recapitulate features previously observed with maternal/zygotic null, it would be really important to be sure there is no off-target effects (since the conclusion are purely genetic epistatic relationship, it seems rather essential). Along these lines, could the authors:
- Check whether some of the specific features of the Rap1 KD phenotype (epithelial rupture, and/or Baz dispersion and/or heterogeneity of cell size) are recapitulated either with another RNAi or best, with the M/Z mutant the authors previously used ? (Choi et al 2013). I suspect one of the reason for not using the M/Z mutant is the ambiguity for the paternal allele which can be either WT or mutant, but observing qualitatively the same phenotype in the "strongly" impaired Rap1M/putativeZ embryo would already be an important validation.

This was an important point. The other available Rap1 RNAi lines are those in vectors tailored for somatic rather than germline expression—we tried one of these and it was not embryonic lethal. Thus we took a different approach to verify that the effects we saw were due to reduced Rap1 function—by expressing the Rap1S17A mutant, which is locked in the GDP bound

conformation (the OFF state) and thus is a well characterized Rap1 dominant negative and one we and others have used in Drosophila. The results were clear— Rap1S17A expression mimicked all of the phenotypes we observed in the Rap1 RNAi embryos. As we now state: "To confirm that the cell shape and epidermal integrity defects we observed were due to knockdown of Rap1 and not to off-target effects, we examined embryos expressing a GDP-locked Rap1 mutant, Rap1S17A, as an alternate way of reducing Rap1 activity. In our earlier work we found that expressing Rap1S17A mimics effects of Rap1M/Z mutants and of Rap1 RNAi on Cno localization and initial AJ positioning during cellularization (Bonello et al., 2018). We thus expressed Rap1S17A maternally and zygotically using the GAL4/UAS system and the matGAL4 drivers. Cuticle analysis revealed that Rap1S17A led to the same strong disruption of epidermal integrity seen in Rap1M/Z mutants (Sawyer et al., 2009) and after Rap1 RNAi (Fig S2A,C vs B,D). We then examined the effects of expressing Rap1S17A on morphogenesis, comparing it to Rap1 RNAi. Rap1S17A expression also blocks mesoderm invagination (Fig S2E, red arrow). At stage 7, we observed the same dramatically altered apical cell shape (Fig S2E, E', and E") that we had observed after Rap1 RNAi (Fig. 1C, 3F). Cno localization was more disrupted than that of Arm (Fig. S2F), but places where Cno was retained also retained elevated levels of Arm (Fig. S2F, arrows), as we observed Rap1 RNAi (Fig. 1C,E). By stage 8 Rap1S17A expression led to unbalanced contractility with hyperconstricted cells ventral to (Fig. S2G-I, brackets) or between the mitotic domains (Fig. S2H,I cyan arrows), epithelial folds (Fig. S2G,J yellow arrows), and gaps appearing at AP borders and rosettes (Fig. S2J red arrows), all defects that matched those seen after Rap1 RNAi (Fig. 4). Embryos expressing Rap1S17A had a similarly disrupted epidermis (Fig. S2K,L vs Fig 5B-D), with only patches of intact epidermis, consistent with their cuticle defects ((Fig. S2A-D). Thus, two different methods of reducing Rap1 activity both lead to major defects in balanced apical contractility and cell shape regulation."

These new data are in new Fig S2. We also further clarified how we had previously verified the effects of Rap1 RNAi when we first introduced the reagent. Finally, we added to the Discussion another point that supports the idea that the effects of Rap1 RNAi are full on-target—namely that in our earlier work we had found that simultaneous over-expression of wildtype Cano can substantially rescue the defects in morphogenesis of Rap1 RNAi, consistent with the idea that activating Cno is a key role of Rap1—however, this did not rescue viability, consistent with Rap1 also having other effectors.

- Similarly, since the authors cannot provide evidence for Dizzy depletion with their RNAi line, and apparently cannot use the maternal zygotic mutant, can they show that the phenotype is indeed rescued by providing a transgene not targeted by the dsRNA ? I suspect this will be difficult since the targeted region is in a codon, but there might be way to solve this using codon degeneration and generating a dizzy transgene poorly targeted by their construct ? (either by transgenesis or just mRNA injection).

These two controls seem quite essential as the phenotype observed are not necessarily that specific of the targeted genes (similar elements can be observed with partial depletion of polarity of adhesive components as previously documented by the authors).

We have addressed questions concerning our dzy RNAi reagent in several different ways. First, as noted in the response to reviewer 1 above, we tried to validate knockdown using a Dzy:GFP line but the signal was too weak and variable to provide confidence that the variability wouldn't bias our results. We obtained another dzy RNAi line from among the NIG lines from Kyoto, and it did not result in embryonic lethality—this was not surprising as this line and others we had previously tried were in UASt vectors that are not well expressed in the germline.

We then took a third approach to reduce the concern that the effect was due to an off-target hit. In our experiments in the original version of the paper, we combined dzy RNAi with maternal heterozygosity and zygotic heterozygosity or homozygosity for the null allele of dzy. We now have compared the cuticle phenotype of embryos in this cross with or without the dzy mutation. Adding the dzy mutation increased the fraction of embryos that have the more severe epidermal disruption phenotypes (in addition to defects in head involution) from 36% to 45% (n≥270 embryos of each genotype). This supports the idea that the defects we see are due to reduction in Dzy expression.

We now also revised and rearranged the text to make clearer what data support the idea this RNAi being a good replicate of dzyM/Z mutants and have included the important caveat that we cannot absolutely rule out off target effects. These extensive changes can be seen on pp. 14-15 of the revised manuscript.

As discussed above, it was not clear to which extend the specificities of Rap1 phenotypes were totally novel. For instance, the heterogeneity of cell apical area was already described in Choi et al 2013 (albeit at earlier developmental stage). Similarly the authors already showed part of the phenotype at stage 7. In a way, it was not necessarily obvious how the previous data were not sufficient to reach the same conclusion about the existence of other Rap1 targets. It might be useful to state more clearly which observations are completely novel (specially based on the numerous previous work from the same laboratory) either in the discussion or in the introduction.

We apologize for our lack of clarity in this regard—this was an important point. Previous analyses of Rap1's roles in embryonic development are essentially confined to the very first two events—polarity establishment during cellularization and ventral furrow invagination. None of the other events of the subsequent many hours of embryonic morphogenesis have been explored in Rap1 mutants or via Rap1 knockdown by RNAi. We have revised the second to the last paragraph of the Introduction and the first paragraph of the Discussion, to more clearly lay out what was known before and what was new.

3. Two important features of the Rap1 phenotype are related to heterogeneity of cell size (which the authors propose to be driven by heterogeneity of tension) at stage 7 and near dividing cells, and early disruption of Bazooka. As mentioned above, at this stage it is hard to know to which extend morphogenesis defects are mostly driven by disruption of adhesion or misregulation of tension (or both). It will probably hard to sort these contributions since there is a lot of mutual regulation, but it might be good to document at least the distribution and

levels of MyoII in these genotypes (comparing Rap1 KD, Canoe mutant and Dizzy KD). This will help to see whether there is a global increase/decrease or just an increases heterogeneity of MyoII between cells. This information might have already been published by the group, and in that case it would be important to include this information in the discussion.

This was an excellent suggestion, and the data obtained strengthened the manuscript. To address the role of myosin-generated tension, we examined the effects of Rap1 RNAi on myosin localization, using strains expressing GFP-tagged E-cadherin and mCherry-tagged myosin. This revealed that Rap1 RNAi led to loss of the tight connection of myosin to AJs at AP borders and at rosette centers, as we had seen in cno mutants, but did not lead to full loss of cortical myosin. This data is in new Figure 7. Future work will be needed to explore how rap1 regulates myosin and apical contractility.

4. If tissue rupture is indeed preferentially formed in regions of high tension, would it be possible to partially rescue this phenotype by a mild reduction of tension? (by reducing Rho and/or ROCK activity either genetically or using low concentration drug injection). Admittedly, this might be a difficult experiment to perform, if the point of the article is purely epistatic it might not be essential for this publication, but it would for sure add some mechanistic explanation. Maybe there is already published data along this line and it might be interesting to discuss it.

This is an interesting issue, but given the many suggestions by the three reviewers, one which we feel extends beyond the scope of the current manuscript. We have explored this issue in our recent work on the cnoΔRA mutant (Perez-Vale et. al 2021), using treatment with the ROCK inhibitor, and surprisingly found that this enhanced rather than suppressed the junctional gap phenotype. In that manuscript, we speculated that this was due to the fact that reducing tension also reduces the tension-dependent strengthening of AJ-cytoskeletal connections. We thus have added the following paragraph to the discussion: "In the future, it will also be important to more directly examine the effects of Rap1 RNAi on the balance of apical contractility, by using approaches like laser cutting to directly measure tension on different borders. In our work on the cnoΔRA mutant (Perez-Vale et. al 2021), we explored whether reducing tension by treatment with a ROCK inhibitor to reduce myosin activation, would reduce or reverse the junctional disruption in the mutant. Surprisingly, this accentuated the junctional gaps seen, rather than reducing them. We speculated that this was due to the fact that reducing tension also reduces the tension-dependent strengthening of AJ-cytoskeletal connections. Similar analyses could be done in after Rap1 RNAi." We also began to address the role of myosin-generated tension by examining the effects of Rap1 RNAi on myosin localization, as described above.

Other minor points :
- So far the authors provide information about canoe planar polarised distribution upon depletion of Rap1 and Dizzy. What about apico-basal distribution and global levels ? Based on previous results from the group, I assumed that Canoe cortical recruitment was clearly reduced

upon Rap1 depletion. Is that the case here as well (specially compared to dzy RNAi and CnoDRA) ?

We're grateful for this suggestion. We added new data in new Figure S1, showing the apico-lateral distribution of Arm and Cno after Rap1 RNAi—neither is dramatically altered. The question of relative cortical levels was also an excellent one. First, we have clarified what we observed in our earlier work—namely that while Cno is lost from the membrane during cellularization in Rap1 mutants, it returns to the cortex as gastrulation starts (Bonello et al., 2018). Next, we used quantitative imaging to compare the levels of Arm and Cno at AJs after Rap1 RNAi. This revealed that levels of Arm were mildly reduced at AJs (mean 73% of wildtype; 12 bicellular borders per embryo in 5 stage 7-8 embryos). Levels of Cno were more substantially reduced (mean=33% of wildtype). This data is also in new Figure S1, and strengthened our mechanistic conclusions.

- Figure 9 : could the authors show the armadillo channel separately to visualise the planar polarity ?

Good suggestion—we have added those panels (now in Figure 10)

- There are some striking similarities between the phenotype of aPKC mutant and Rap1 depletion (see notably the higher variability of cell apical area already described in Choi et al 2013). Along the point in the discussion about the effect of partial depletion of aPKC (results for Eurico Moraes de Sa lab), this might be an interesting element to add.

This was an excellent suggestion. We have expanded this paragraph in the Discussion to include these ideas. This work also prompted us to carry out a preliminary examination of the effect of Rap1 RNAi on aPKC localization. This confirmed that aPKC cortical localization is retained after Rap1 RNAi. This data is included in new Fig. 7. As we note in that section of the results, and in the revised Discussion, it will be important in the future to determine whether Rap1RNAi simply reduces rather than eliminates aPKC localization or activity.

Reviewer #3 (Remarks to the Author):

In this manuscript, Perez-Vale, Yow, and the team investigated how Canoe/Afadin, which is associated with adherens junction, a small GTPase Rap1, and a GEF regulators Dizzy, influence each together during early Drosophila embryogenesis. To this end, the authors compared different phenotypes, including planar polarity at the cell-cell junction, cell shape, and tissue integrity at different embryogenesis, between wildtype and mutants, including Rap1 RNAi, cno mutant, and dzy RNAi. Depending on the similarity/difference of phenotypes in each fly line, the authors characterize whether Dizzy regulates Canoe via Rap1 or whether other GEFs play roles in regulating Canoe through Rap1. The authors concluded that this is context-dependent. Overall, this study is addressing important but challenging questions. Most of the data and interpretations are solid and intriguing (except a few I highlight below), and I would support publication in MBoC provided some of the concerns I list below can be addressed.

We appreciate these supportive comments and the exceptional careful read of the manuscript and very thoughtful suggestions

Major comments:
#1. I feel that the first half of the result section, which is characterizing the phenotype of Rap1 RNAi (Figs. 1-5), is hard to follow. Similar to what the authors did at the begging of the second half of the results using dzy RNAi (Figs. 6-10), it would be helpful if the authors state at the beginning of this result section (page 5 middle) that:
- The first goal is to determine which Rap1's roles during embryogenesis require the Rap1 effector Cno, and which ones don't.
- To this end, the authors compared different phenotypes, including planar polarity at the cell-cell junction, cell shape, and tissue integrity at different embryogenesis, between wildtype and mutants, including Rap1 RNAi, cno mutant.
- The authors envisioned two scenarios. The first group of Rap1-dependent morphological characteristics is Cno dependent. In this case, Rap1 loss would mimic the loss of Cno. The second group is Cno independent.
- "explore the roles of Rap1 and its **regulators** during morphogenesis" (page 5 middle) sounds confusing. I believe this section is not to investigate the upstream of Rap1, i.e., regulators, but the downstream effects.
These lines could help the reader understand the structure and idea behind the lengthy result section (Figs. 1-5).

These were GREAT suggestions! We have completely revised the first section of the Results section (and also revised the last section of the Introduction) to include these suggestions. For example:

"Our first goal was to define the roles of Rap1 during morphogenesis and to determine which of these roles require its effector Cno and which might involve other effectors. To do so, we compared the effects of Rap1 RNAi to those of loss of Cno, which we and others had previously characterized. We examined cell shape, cell rearrangements, cell junction stability under tension, junction protein planar polarity and overall tissue integrity from the onset of gastrulation to the completion of dorsal closure. We envisioned two possibilities: 1) Rap1 RNAi precisely mimics loss of Cno, suggesting Cno is its only effector during these events, or 2) Rap1 RNAi affects additional cell biological events, suggesting the existence of other important effector(s)."

#2. The first section of the results "While Rap1 RNAi mimics the effect of Cno loss on AJ planar polarity..." is confusing.

- I suggest citing their own data, including the distribution of Cno, instead of simply citing the previous paper, in the first paragraph of page 6.

Good suggestion—we added references to Fig. 1B

- I suggest adding Pyd images in Fig.1. It looks strange not to show the images but provide the quantifications in Fig. 2B.

This was an excellent suggestion—we have added the requested images and they are very helpful as they clearly illustrate the elevated planar polarity of Pyd which is quantified in Fig 2.

- "Bazooka (fly Par3; Baz) is obviously planar polarized at this stage..." It would be helpful to specify Bazooka is more on DV borders than AP borders, instead of "planar polarized".

Fixed

- It is not clear whether the 4-line-long segment starting with "In contrast, Cno loss enhances..." represents the results from this study or the findings from the other papers. Please cite the data or the references.

Good suggestion—fixed

- What "Cno loss" means? Are you referring to cno mutant? Please specify.

Fixed—this section now reads: "Previous work revealed that in cno null mutants or after strong cno RNAi the planar polarization of Arm, Pyd and especially Baz is enhanced, by strongly reducing their accumulation on AP borders (Sawyer et al., 2011; Manning et al., 2019). This weakens AJ-cytoskeletal connections there, leading to apical gaps at AP borders and TCJ (Sawyer et al., 2011; Manning et al., 2019)"

"by strongly reducing their accumulation on AP borders." How do you know this is only due to the reduction at AP borders? How about the accumulation at DV borders? Or the combination?

With the text revisions mentioned above, we have clarified that this was documented in earlier work and the citation is now clearer.

- "core AJ proteins remain localized to AJs in cno mutants." Which data shows this?

We have added the appropriate citations here

- "While Arm and Cno return to apical AJs..." Where do Arm and Cno return from?

Thanks for pointing out places where we assume knowledge most readers will not have! This is now revised as follows: " While Arm and Cno are either basally mislocalized (Arm) or lost from the membrane (Cno) during cellularization in Rap1 RNAi embryos, both relocalize to apical AJs as gastrulation begins (Bonello et al., 2018)".

- "places where both Arm and Pyd were also enriched (Fig. 1E", inset)." I suggest adding a rectangle representing the same ROI in E'. Moreover, a red arrow is missing in E'.

Good suggestion and remarkably careful looking at the Figure! Fixed.

- When explaining the changings in planar polarity shown in Fig. 2A-D, the authors used the terms "elevating", "reversed", and "enhanced". I suggest explicitly explaining 1) "reversed" represents the localization change from AP>DV to DV>AP, and 2) "elevating" and "enhanced" represent the changes from AP>DP to AP>>DP.

Good suggestion. We have clarified that section as follows: "Rap1 RNAi mimicked the effect of loss of Cno on Arm and Pyd localization (Sawyer et al., 2011; Manning et al., 2019), enhancing planar polarization of Arm (Fig. 2A) and Pyd (Fig. 2B) by increasing their enrichment on DV borders. Rap1 RNAi also reversed the planar polarity of Cno (Fig. 2C), altering mild enrichment on AP borders to strong enrichment on DV borders. "

#. "Together these data suggest Dzy function is... but is important to reinforce AJs under elevated tension, like TCJs and constricting AP borders. (page 13 bottom)". Please specify which data support the statement "Dzy function is important for constricting AP borders"?

We have clarified the reasoning for this as follows: "Together these data suggest Dzy function is dispensable for Cno and Arm localization to AJs, but is important to reinforce AJs under elevated tension, like TCJs and constricting AP borders, as reflected by the gaps that appear at these locations after dzy RNAi (Fig. 8G, I)."

#. I feel the statement "Dzy is required for ... Cno tension sensing" is a bit of a stretch. The authors show "Cno TCJ enrichment was strongly reduced after dzy RNAi (Fig. 8A vs B)". However, the reduction of Cno at TCJ does not simply mean the tension sensing via Cno is compromised. To make this claim, junctional tension needs to be measured (for instance by laser ablation). Alternatively, the authors can simply highlight the role of Dzy in Cno localization at TCJ.

Good point. We altered this section heading to "Dzy is important for maintaining most but not all aspects of AJ planar polarity and for Cno recruitment to TCJs" and made sure that the following paragraphs also did not overclaim.

Minor comments:

#. "horizonal borders" can be replaced with "DV borders".

Good point--We have clarified this in the Methods

#. I feel there are too many "dramatic", "striking", or "drastic" observations.

Guilty—we removed eight instances of dramatic, one instance of drastic, and three of striking.

#. I do not see "fold inward (page 8 top)" in Fig. 3D.

We have softened this statement

#. I do not see "and AJ gaps at AP borders and TCJs (page 8 top)" in Fig. 3G. Adding arrows should be helpful.

Arrows added

#. It would be informative to quantify the gap in Rap1 RNAi (Fig. 3), similar to Fig. 8I.

Because of the severity of the Rap1 phenotype and the infolding in many places, we chose not to quantify this as we think the comparison would be parallel.

#. "Fig. 8D, E, J, yellow arrows (page 13 middle)". There is no Fig. 8J.

Fixed—thanks! Should have been 8D, E, G

RE: Manuscript #E22-05-0176R
TITLE: "Rap1 regulates apical contractility to allow embryonic morphogenesis without tissue disruption and acts in part via Canoe-independent mechanisms"

Dear Mark,

Thank you for your revised manuscript, which has now been seen by the reviewers. As you can see, all is now in order and I am happy to accept your MS for publication in MBoC. I would also thank you and your colleagues for all your efforts to respond to the feedback. (Something that I think the reviewers also clearly appreciate.)

Best wishes,
Alpha

Alpha Yap
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Peifer:

Congratulations on the acceptance of your manuscript.

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

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

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The authors have convincingly and comprehensively addressed all my concerns (and in my view, there was also a major effort that addressed the concerns of the other reviewers). The new data have significantly improved the manuscript, which now represents a solid work advancing our understanding of the regulation and function of small GTPase Rap1 during morphogenesis.

Minor point:

In Abstract, "constriction-in its absence" . Please confirm if the long hyfen is there on purpose.

Reviewer #2 (Remarks to the Author):

The authors have very significantly improved the manuscript, providing new controls, better explaining the novelty and the rational of the study and clearly outlining the similarity and differences between phenotypes. The modification of the text and the figures also make the reading much easier, clarify the rational and clearly mention relevant controls that were previously performed as well as the limits of the previous published studies.

I fully support publication without changes.

I just noticed a little typo end of page 7 ("Rap1 regulates apical poisoning of AJs during cellularisation". I guess the authors meant "positioning")