# Interplay between actomyosin and E-cadherin dynamics regulates cell shape in the *Drosophila* embryonic epidermis

Joshua Greig and Natalia A. Bulgakova
DOI: 10.1242/jcs.242321

**Editor**: Kathleen Green

**Review timeline**

| | |
|---|---|
| Original submission: | 28 November 2019 |
| Editorial decision: | 28 January 2020 |
| First revision received: | 11 May 2020 |
| Editorial decision: | 19 June 2020 |
| Second revision received: | 24 June 2020 |
| Accepted: | 1 July 2020 |

---

**Original submission**

First decision letter

MS ID#: JOCES/2019/242321

MS TITLE: Interplay between actomyosin and E-cadherin dynamics regulates cell shape in the Drosophila embryonic epidermis.

AUTHORS: Joshua Greig and Natalia A Bulgakova
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. I would refer you to the referees' detailed comments for issues you will need to address in order to make the paper publishable. In particular, note comments from the third referee, that the paper would benefit from validation of the more novel aspects related to the p120-Arf1 arm of the proposed model by carrying out rescue experiments and determining why overexpression rather than knockdown of p120 influences cell shape.

If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

The manuscript of Greig and Bulgakova uses the Drosophila embryo to address how epidermal cell shapes are influenced through the dynamics of E-cadherin and actomyosin complexes. Actomyosin dynamics is seen to suppress E-cadherin endocytosis and cell elongation in response to upstream RhoA signaling while clathrin-mediated endocytosis of E-cadherin, promoted by the Arf1 GTPase, supports elongation.
Interconnections are observed between these two determinants of cell shape, given observations that RhoA reduces Arf1 recruitment to the plasma membrane, and that p120ctn promotes both Arf1 and RhoA functions.

Overall, the work is of interest to cell biologists interested in determinants of cell shape, and thus morphogenesis. It does provide support for the interplay of cell-cell adhesion and actomyosin contractility and the role of p120ctn in both. It also provides additional evidence that in keeping with vertebrates Drosophila p120ctn indeed has developmental roles, following earlier reports from an earlier group that it may be minimal or masked by other mechanisms.

*Comments for the author*

This manuscript is complicated to follow when relying on the text/ figures alone, but thankfully a model is offered in Figure 7 that can be used in conjunction. The model is generally consistent with the authors observations, that take advantage of various available Drosophila mutants, whose use/ testing was rationalized based upon prior published reports.

For example, a rationale for the evaluation of Drosophila p120ctn in the context of cell shape included note that members of the p120ctn family modulate small-GTPases as well as the cadherin-catenin complex and thus provide "good candidates to mediate the interplay between cortical tension and cell adhesion" (Page 8). Likewise, on Page 14, one rationale for examining Arf1 was that it is known to be involved in various forms of trafficking and that "The GTPase Arf1 has been reported to interact with E-cad and other AJ components (Shao et al., 2010; Toret et al., 2014). Therefore, we examined if Arf1 acts downstream of p120ctn . . .". No screens are employed nor biochemistry to test for direct or indirect interactions of the components examined. Rather, genetics is used in conjunction with various assay readouts, from phenotypes (e.g. cell aspect ratios), cortical tension/ recoil measurements, protein localization/ GFP intensity at cell-cell borders, etc. As the authors point out, the interplay of the components is complex and we are seeing only a small part of the larger picture here, based upon the authors selection of available mutants of interest. The UAS/ GAL4 system was used to facilitate the overexpression or removal of a gene product of interest in defined cells within each segment.

Overall, the work is of interest to cell biologists interested in determinants of cell shape, and thus morphogenesis. It does provide support for the interplay of cell-cell adhesion and actomyosin contractility and the role of p120ctn in both. It also provides additional evidence that in keeping with vertebrates Drosophila p120ctn indeed has developmental roles, following earlier reports from an earlier group that it may be minimal or masked by other mechanisms.

Questions & Comments:
This reviewer did not find a point-by-point response to the prior reviewer comments, only a very brief response dealing largely with reference to the text. The authors state "As the function of p120ctn is not the main focus of this new manuscript, but rather it is used to investigate the broader question of cell shape, we feel that some of the reviewers comments from our previous submission are no longer relevant to this submission". This reviewer re-reviewed the manuscript.

Figure 3 indicates that in the "p120ctn mutant" embryo, MyoII-YFP was reduced specifically at the AP borders. Why is it then that a change in aspect ratio was not observed in Figure 1 in the p120ctn mutant?

Page 9. It is confusing to refer to kinase dead Venus-RokK116A as Rok-Venus, since the latter does not convey that it is a kinase dead mutant of Rok. Better to use the full nomenclature of Venus-RokK116A, or a name such as Rok-KD-Venus.

In Figure S1, the quantitation appears to have the labels reversed for p120-/- and p120 over expression.

In Figure 2, need to make color keys larger in 2H. Likewise in Figure 3J&K. The label "C" is missing in Figure 5.

Figure 4B&C, why do p120ctn overexpression and p120ctn mutant have the same effect on FRAP of E-GFP?

Is there an accessible way in this system to look at RhoA activation more directly? For example, with biosensors?

Are there straight-forward ways to manipulate the levels of E-cadherin more directly in the cells of interest, to evaluate the impact upon aspect ratios? For example, does removing one allele for E-cadherin have an effect, or mild overexpression?

More caution needs to be exercised in the wording with respect to the term "RhoA signaling". Be more specific/ spell out what is meant for each context under discussion.

Can biochemistry be undertaken to establish the relationship of p120ctn with Arf1 in this system? For example, does the absence of p120ctn have an impact upon Arf1 association with the larger cadherin-catenin complex? Or genetically, does overexpression of a p120ctn mutant that is incapable of binding cadherin have the same or a different phenotype relative to p120ctn?

Comment upon the DV border under compression. How does this fit with other studies, and how does it come about?

Page 10 last sentence of final paragraph, why is it said that p120ctn loss results in a decrease of E-cad-GFP at both AP and DV borders (since one would expect reduced endocytosis and thus enhanced E-cad levels)?

Bottom of Page 11, Why is E-cad-GFP less dynamic in p120 overexpressing cells when considered in relation to the model in Figure 7, where it would seem that p120ctn overexpression would be expected to enhance endocytosis?

What about p120ctn effects upon Rac? There is little to no discussion of Rac.

Page 14, how is it that "The loss of p120ctn resulted in a uniform decrease in the amount of Arf1-GFP at both the AP and DV borders", when the loss of p120ctn also reduced E-cadherin at both the AP and DV borders. One might think that a reduction in Arf1 function/ endocytosis, would expect to correlate with an increase in E-cadherin levels?

Page 19, Confusing when use the term "stabilization" in isolation (to mean the immobile fraction as discerned by FRAP) since many readers will instead interpret "stabilization" as meaning an increased level of total protein. Better to spell it out and note that you are referring to the immobile fraction.

Reviewer 2

*Advance summary and potential significance to field*

In this work, Greig and Bulgakova study the interplay between junctional proteins including E-cadherin and associated p120 and Myosin II regulators in the context of epithelial cell shape changes during Drosophila embryonic development. The authors focus on determining junctional aspect ratios of distinct cell borders localisation of relevant proteins at cell-cell junctions, tension

measurements using laser ablations and FRAP measurements under different conditions (overexpression/knockout). The authors conclude that there is a dual function of p120 in regulating distinct cell borders, which is dependent on two distinct GTPase signalling pathways, RhoA and Arf1. This study extends previous observations (Bulgakova 2013, 2016) and is of potential significant interest to readers in the field and beyond for investigators working on tissue morphogenesis, adhesion and actomyosin regulation.

*Comments for the author*

However, the authors fail to provide sufficient evidence and clarifications in multiple aspects of this work which makes their conclusions quite speculative. Following concerns should be addressed to make the present work publishable in Journal of Cell Science:

Major concerns:
1) The contribution of adhesion and tension in differential regulation of AP and DV cell shape changes needs clarifications and strengthening.
a) The authors delineate proteins and pathway that might regulate cell shape, but how up or downregulation of these components specifically affect AP and DV junctions is not clear from the aspect ratio measurements alone. Similar changes of aspect ratios can be achieved by multiple ways (such as shortening of DV borders with same AP length, or elongation of AP borders with same DV length). Given the different localisation of proteins at different borders, the specific impact on AP/DV junctions should be indicated where cell shape changes were measured.
b) From the presented images (eg. Fig. 2A, D), knockout of p120ctn seems to produce more elongated cells (along AP border) compared to control cells. It is surprising that the aspect ratio when quantified did not change (eg. Fig 2C). As overexpression of p120ctn seems to result in less elongated cells (Fig. 2A, B) it would be logical to expect the opposite effect in the knockout situation. How do the authors explain this discrepancy?
2) 2 photon laser ablation has previously successfully be used to determine tension of epithelial junctions in Drosophila, However, the tension measurements presented here seem currently very superficial.
a) Due to the low quality images from the laser cuts it is not clear where the cuts are made and how the junctions/vertices behave after severing. A better representation of the laser ablation experiments such as a kymograph should be included, the dual-colour overlay is very hard to interpret, especially at the DV borders.
It would also help to plot distance over time (or speed) to visualise the recoil dynamics.
b) Further, the accumulation of what looks like a cavitation bubble at the junctions after ablation is visible (Fig. 1G, 2G) and indicates a wound healing response rather than a clean junctional cut. A control for leakage due to introduction of a wound should be included (cytoplasmic fluorophore) to exclude cell membrane damaging.
c) Laser ablation is commonly used to measure line tension in epithelial cells, where recoil indicates outward directed tension before ablation due to force balance of junctional tension at the vertices (eg. Rauzi and Lenne, Curr Top Dev Biol 2011). How do the authors explain measurements of compression with laser ablation? Compression would probably result in buckling of junctions after ablation, which would make it hard to interpret vertices movements after tension release. This is to my knowledge a new approach and needs explanation. What model could be used here to describe the ratio measurements of cortical forces? A graphical illustration would help to understand the rationale.
d) For reproducibility, the authors should include the measured time points for each ablation experiment, and in the methods section the physical parameters (pulse duration, power density) and the method used to track the vertices.
3) The FRAP measurements are a very useful tool to measure dynamics of junctional proteins. However following aspects need clarifications/considerations:
a) It would be valuable for the reader to show images of the bleached area before/at/after bleaching to visualise where and how much of the junctions were bleached. Also, an indication of the recorded time points measured should be mentioned in the text or/and in the figure legends.
b) The authors use the FRAP measurements to exclusively determine mobile/immobile fraction to estimate endocytic recycling. An important component of cell-cell adhesion regulation is dynamics of E-cadherin by diffusion of molecules from/to and within the cell membrane, especially in the context of cortical actomyosin.

As the authors already have the FRAP measurements in hand, it would be valuable to determine the half-life of the recovery for the different experiments to consider factors independent of endocytosis.
c) The plateaus are not visible in most graphs, the recovery seems still ongoing. To clearly indicate saturation the plots should be extended to plateau level.
4) Using CLC FRAP measurements as a proxy for E-cadherin dynamics is an oversimplification, as regulation of E-cadherin endocytosis was shown to occur also in a clathrin-independent way such as through caveolin-mediated internalisation or lipid raft-mediated endocytosis. Therefore it would make interpretations difficult and might most likely also affect important parameters, for example the time scale of endocytosis as can be seen in Fig. 4B,C vs Fig. 3J, K. Also, from Fig. 4B,C it is not clear if AP or DV borders were measured. Instead of using this indirect approach, I was wondering why the authors did not continue measuring E-cadherin dynamics directly (as done in Fig. 3), which would give a much better indication of the process.
5) The link between p120-E-cad-RhoA is intriguing, but needs to be better supported:
a) The authors claim that dn-Rho seems to reduce intensities of E-cad at AP borders. From the images (eg. Fig. 4D) it seems that E-cad localisation changes from continuous decoration along AP borders (control) to spot accumulations, which indicates a reorganisation of E-cad along the junctions, rather than increased endocytosis.
b) The authors explore a possible link between E-cad and RhoA in Fig. 4, but their conclusions are related to the role of p120 in Rho signalling ("In cells overexpressing p120ctn, the increase of E-cad at AP borders was accompanied by increased activation of RhoA"). In fact, there is not a single experiment that shows what happens to p120 under dn-Rho and ca-Rho conditions and vice versa and the conclusion remains speculative.
The authors mention that p120 has a tension-dependent function, but direct evidence is missing.
c) From the FRAP measurements in Fig. 4I it is not clear if DV or AP borders were measured. Is there any change in dynamics in the case of dn-Rho?
d) As mentioned in point 4 above, instead of measuring CLC dynamics (Fig. 4), FRAP on E-cad should be performed, which would give a direct readout for E-cad dynamics and would clearly indicate how elevated and reduced levels of RhoA control E-cad turnover at AP/DV borders.
6) The authors mention that RhoA prevents the localisation of Arf1 at the plasma membrane (Fig. 7B), but no direct evidence is shown. For example, what happens to Arf1 in ca-Rho expressing cells?
7) Fig. 4F and Fig. 6C seem identical.

Minor points:
1) As the authors indicate actomyosin as major regulator, it would be valuable to investigate if also actin localisation is anisotropic (like Myo II).
2) The cell shape change called "cell rounding" is misleading and should be changed, as this terminology is commonly used for cells rounding up due to mitosis. The presented cells in the epithelia after manipulations appear at best less elongated (eg. Fig. 2A), but not round.
3) The graphs are often not aligned with the images, which makes it difficult to find the corresponding graph amongst all the panels within the figures. This should be adjusted.
4) Fig. 3D: The E-cad levels at the DV borders show a very high standard deviation compared to AP and need more data points to clearly demonstrate no significance.
3) Fig. 5F: It seems as there are 3 curves plotted, I believe there should be only 2.

Reviewer 3

*Advance summary and potential significance to field*

This manuscript by Greig and Bulgakova examines the interplay between adhesion and contractility in the regulation of cell shape in the late Drosophila embryonic epidermis. Using genetic perturbations that modulate adhesion dynamics and/or actomyosin contractility, they argue that the acquisition of the anisotropic and elongated cell shape is the result of the "counteracting" effects of actomyosin contractility that suppresses cell elongation, and adhesion that promotes it. They arrive at these conclusions through the analysis of cell shape contractility and adhesion dynamics in regulators of either or both. Specifically, they examine the effects of altering the levels p120catenin- a known regulator of adhesion dynamics, Rho GTPase signaling which influences both adhesion and contractility, and the Arf1 GTPase that they show here influences adhesion dynamics downstream of p120 catenin. They propose a model in which they suggest that at the long

interfaces of epidermal cells, p120 catenin activates both Rho and Arf GTPases to respectively regulate contractility and adhesion dynamics, with Rho activation also inhibiting Arf1 GTPase.

That contact expansion requires the downregulation of contractility has been established in many systems. It has also been demonstrated that in elongated cells with anisotropic shapes the distribution of myosin and ECadherin/Bazooka are complementary. What molecular mechanisms mediate these distribution patterns, how they may influence one another and whether these patterns govern the acquisition of anisotropic shapes are challenging questions that this manuscript addresses, using p120catenin (which one of the authors had previously demonstrated modulates ECadherin dynamics by specifically affecting the mobile ECadherin pool that is in a complex with Bazooka) as its center-point.

*Comments for the author*

Although the work presented in this manuscript demonstrates the reciprocal and anisotropic effects of upregulating or downregulating p120 levels on actomyosin contractility and ECadherin levels, their effects on Ecadherin dynamics (increase and decrease respectively), the effects on clathrin dynamics and Arf GTPase distribution in the two perturbations are similar. Also, while p120 overexpression leads to a reduced anisotropy in cell shape, loss of p120 function has no effect on shape. To substantiate the model they propose, the authors examine the consequences of reduced or increased contractility (using Rho/RhoGEF2 knock down/activation) and identify effects on Ecadherin distribution and clathrin dynamics. Since most of the conclusions made are based on the similarities in the phenotypes of different classes of perturbations, some of the statements made (eg: ECadherin was also stabilized when Rho signaling was downregulated in p120 mutants, p14; and we have demonstrated that p120 regulates actomyosin dynamics via RhoA signaling and ECadherin dynamics via both RhoA and Arf1; p16) are too strong and must be validated by genetic rescue experiments. In the only rescue experiment presented in this paper, the authors show that activating Arf1 in a p120 mutant restores clathrin dynamics to wildtype levels. While this illustrates a role for Arf1 in regulating Ecadherin dynamics, the influence of p120 dependent Ecadherin dynamics on cell shape anisotropy remains unclear. Also, while the results demonstrate that cell shape is influenced by regulators of adhesion and contractility, they also reveal that the picture is complex. In their present form, the experiments do not provide a clear mechanistic picture. Overall, I think the novelty of this paper resides in the demonstration of the requirement of Arf in regulation of anisotropic shape (Fig.S4), its modulation of clathrin dynamics, and the (similar) modulation of its levels by both p120 overexpression and knockdown. I am of the opinion that the paper will benefit from validating one or both arms of the proposed model through rescue experiments that examine the effects of specifically modulating either Ecadherin dynamics or contractility on cell shape changes induced by p120 overexpression or Arf1 knockdown or identifying why overexpression rather than knockdown of p120 influences cell shape. Might p120 levels under tight regulation?

Other concerns:
In Fig.1, the images do not clearly show the effects of ablation. It would desirable to show a time series. In p120 loss of function mutants, were embryos examined for phenotypes in the engrailed domain only? How do the authors explain the lack of effect of p120 reduction on cell shape?
In Fig.5, single images would be helpful to visualize effects on the membrane distribution of Arf1. In the projections, no membrane localization is evident. It would be useful to have a table summarizing the effects of various perturbations tested on the parameters analysed.
The legend to Supplementary Figure 4 does not correspond with the figure supplied. Some panels are missing.

**First revision**

Author response to reviewers' comments

**Reviewer 1**
Advance Summary and Potential Significance to Field:
The manuscript of Greig and Bulgakova uses the Drosophila embryo to address how epidermal cell shapes are influenced through the dynamics of E-cadherin and actomyosin complexes. Actomyosin dynamics is seen to suppress E-cadherin endocytosis and cell elongation in response to upstream RhoA signaling, while clathrin-mediated endocytosis of E-cadherin, promoted by the Arf1 GTPase, supports elongation. Interconnections are observed between these two determinants of cell shape, given observations that RhoA reduces Arf1 recruitment to the plasma membrane, and that p120ctn promotes both Arf1 and RhoA functions.

This manuscript is complicated to follow when relying on the text/ figures alone, but thankfully a model is offered in Figure 7 that can be used in conjunction. The model is generally consistent with the authors observations, that take advantage of various available Drosophila mutants, whose use/ testing was rationalized based upon prior published reports.

Overall, the work is of interest to cell biologists interested in determinants of cell shape, and thus morphogenesis. It does provide support for the interplay of cell-cell adhesion and actomyosin contractility, and the role of p120ctn in both. It also provides additional evidence that in keeping with vertebrates, Drosophila p120ctn indeed has developmental roles, following earlier reports from an earlier group that it may be minimal or masked by other mechanisms.

We would like to thank this reviewer for finding our manuscript of broad interest to cell biologists and are glad that the review found the diagram offered in Figure 7 (now Figure 8) helpful.

Reviewer 1 Comments for the Author:
Figure 3 indicates that in the “p120ctn mutant” embryo, MyoII-YFP was reduced specifically at the AP borders. Why is it then that a change in aspect ratio was not observed in Figure 1 in the p120ctn mutant?

This question was raised by all three reviewers and thus highlights an important point. When we first measured the aspect ratio of the cell in the *p120ctn* mutant we used all the cells in the imaged area (both *engrailed*-positive and -negative compartments) as the organism is a genetic null. This is in contrast to the other experiments presented in the work in which transgenes were only expressed in the *engrailed*-positive compartment of the epidermis. Having compared these compartments, we have detected a significant difference in their cell morphology in the wild-type control embryos (see new Figure S1A). This innate difference, and thus an increased variance, could account for the lack of detected change in the aspect ratio of the *p120ctn* mutant presented in the original manuscript. Now, we have repeated the experiment with the *p120ctn* mutant embryos but only measured the *engrailed*-positive compartment. We indeed detected a significant decrease in the aspect ratio of *p120ctn* mutant cells.

This finding demonstrates the importance of accounting for morphological differences between compartments. We have replaced the measure of aspect ratio for the mutant in Fig. 3 with the corrected graph and have amended the manuscript accordingly in the revision (page 8). We thank all three reviewers for their observation and for drawing our attention to this point.

Page 9. It is confusing to refer to kinase dead Venus-RokK116A as Rok-Venus, since the latter does not convey that it is a kinase dead mutant of Rok. Better to use the full nomenclature of Venus-RokK116A, or a name such as Rok-KD-Venus.

We agree with this comment and have replaced Rok-Venus by $Rok^{KD}$-Venus throughout the text.

In Figure S1, the quantitation appears to have the labels reversed for p120-/- and p120 over expression.

We have corrected the labels in Figure S1.

In Figure 2, need to make color keys larger in 2H. Likewise in Figure 3J&K. The label “C” is missing in Figure 5.

We have corrected the colour keys in these figures and added the label.

Figure 4B&C, why do p120ctn overexpression and p120ctn mutant have the same effect on FRAP of E-cad-GFP?

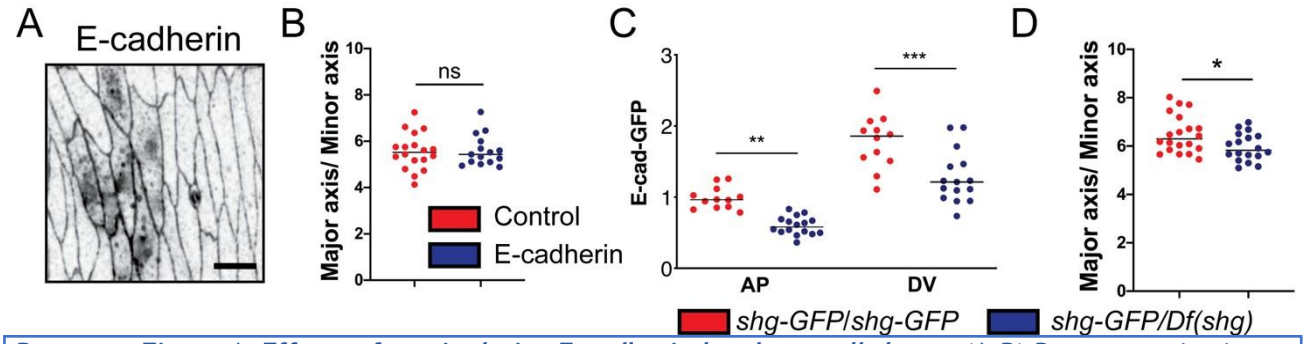
This is an important and interesting point, which is likely to be explained by a combinatorial action of p120ctn and RhoA on Arf1 recruitment to the plasma membrane. Indeed, as we demonstrate in Figure 5F-G, restoration of Arf1 function in p120ctn mutants is sufficient to restore CLC-GFP dynamics as a proxy for E-cad dynamics. We had added the following section in the first paragraph of the discussion to address this phenomenon:
*“As a result, both the depletion and overexpression of p120ctn lead to an increase of immobile E-cad at the cell surface: depletion is likely to do so through directly limiting Arf1 recruitment to the plasma membrane, while overexpression does so through elevating RhoA activity, which then inhibits Arf1.”*

Is there an accessible way in this system to look at RhoA activation more directly? For example, with biosensors?

We agree that this is an important control for our use of MyoII-YFP and Rok$^{KD}$-Venus as readouts of RhoA activation. We have therefore analysed the localization of RhoA biosensor – RhoAGTP-binding domain of anillin (RBD-GFP, Munjal et al., 2015) in embryos overexpressing p120ctn. Although expression of this biosensor produces less clear enrichment at the plasma membrane in comparison to MyoII-YFP and Rok$^{KD}$-Venus, likely because of high cytoplasmic signal due to potential overexpression, the results overall mimics those produced by other methods: enrichment of RBD-GFP at the AP borders relatively to DV, and increased accumulation of RBD-GFP specifically at the AP borders in cells overexpressing p120ctn. We have included the description of this result in the text alongside MyoII-YFP and Rok$^{KD}$-Venus on page 9, and added representative images and graphs to Figure S1F-G.

Are there straight-forward ways to manipulate the levels of E-cadherin more directly in the cells of interest, to evaluate the impact upon aspect ratios? For example, does removing one allele for E- cadherin have an effect, or mild overexpression?

This is an interesting point. We have indeed overexpressed E-cad in these cells using the GAL4/UAS system and found that the cells compensate for elevated transcription of E-cadherin (Response Figure R1A): E-cad overexpression results in only a moderate increase of E-cadherin at sites of cell-cell adhesion (Response Figure R1A), which might be more dynamic as inferred from the elevated intracellular accumulation of E-cad. This compensation seems to nullify the effect of overexpression of full-length E-cad leading to the same aspect ratio as in control (Response Figure R1B). At the same time removing one copy of E-cad does reduce its junctional levels (Response Figure R1C-D), which surprisingly leads to reduced aspect ratio (Response Figure R1E). We should admit that we do not know how such reduction in E-cad copy number and junctional levels alter E-cad dynamics, p120ctn levels, RhoA signalling, and Arf1 recruitment to the plasma membrane. One speculative explanation for this result could be that reduced junctional levels of E-cad lead to reduced p120ctn recruitment, which in turn reduces the recruitment of Arf1 to the plasma membrane but is not sufficient to affect actomyosin levels. This complex interplay between E-cad levels, its trafficking, actomyosin, and cell shape is beyond the focus of the current study but is a fascinating direction for future research.




**Response Figure 1. Effects of manipulating E-cadherin levels on cell shape.** (**A-B**) Representative image (**A**) and aspect ratio (**B**) in cells overexpressing E-cadherin. (**C-D**) E-cadherin levels (**C**) and aspect ratio (**D**) in cells lacking one copy of *shotgun* (*shg*) gene encoding for E-cadherin. Scale bar – 5 µm. * – p = 0.02, ** – p = 0.001, *** – p < 0.0001. ns – not significant.

More caution needs to be exercised in the wording with respect to the term "RhoA signaling". Be more specific/ spell out what is meant for each context under discussion.

We appreciate the reviewer raising a valid concern. We have now provided more direct evidence that p120ctn regulates RhoA signalling as we describe above using the activity biosensor. This makes us more confident in using the term "RhoA signalling" rather than, for example Rok localization alone. Additionally, as localization and its changes of MyoII-YFP, $Rok^{KD}$-Venus, and RBD-GFP mimic each other, we are confident that in other cases, when RBD-GFP was not directly analysed, changes in two others reflect effects on RhoA signalling. However, we have altered the wording to be more specific in cases when RhoA signalling was assessed only based on indirect readouts, but not using the biosensor, or altered through direct or validated manipulations of RhoA activity, e.g. using $RhoA^{CA}$.

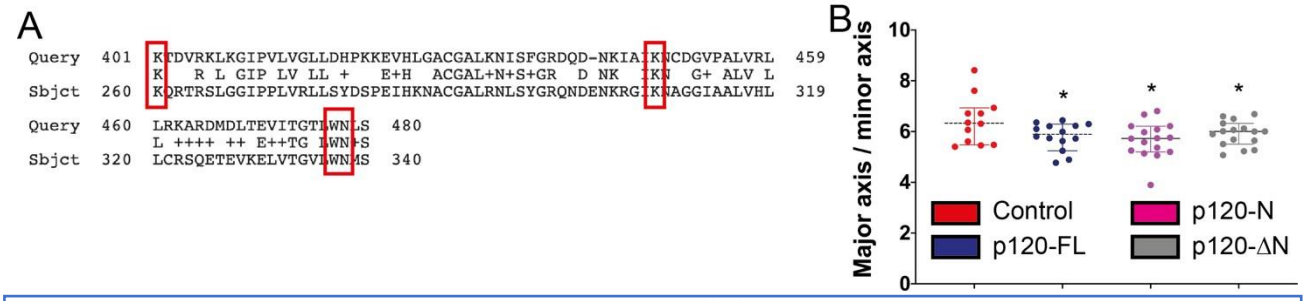
Can biochemistry be undertaken to establish the relationship of p120ctn with Arf1 in this system? For example, does the absence of p120ctn have an impact upon Arf1 association with the larger cadherin-catenin complex?

We did try to co-immunoprecipitate p120ctn with Arf1, however such experiments are challenging in *Drosophila* embryos and were not successful. This does not exclude a physical interaction between them and could be explained by: the poor binding of the p120ctn antibody as shown in Figure S2, the interaction being transient, or it being mediated through p120ctn interaction with an ArfGEF. We think that the discovery of the network interaction between p120ctn and Arf1 is a valuable finding for the field, as highlighted by reviewer 3, and future work can address the exact molecular mechanism of this interaction.

Or genetically, does overexpression of a p120ctn mutant that is incapable of binding cadherin have the same or a different phenotype relative to p120ctn?

We report that the overexpression of p120ctn produces a phenotype predominantly through hyperactivating RhoA, which in turn prevents Arf1 recruitment to the plasma membrane. Indeed, we can rescue the cell elongation defects caused by p120ctn overexpression by simultaneously downregulating RhoA using RhoGEF2 RNAi (see Figure 7). The amino acid residues K401, K444, W477, and N478, which are required for E-cadherin binding in human p120ctn (Ishiyama et al., 2010), are conserved in *Drosophila* (Response Figure R2A). We would envision that the overexpression of a construct, in which these residues are mutated, especially in the presence of endogenous p120ctn capable of E-cad binding, would produce a similar phenotype to those of full- length p120ctn due to its maintaining: a) the ability to bind RhoA through two Rho-binding domains, and b) retaining its plasma membrane recruitment through palmitoylation, which was reported for $\delta$-catenin at the C-terminus (Brigidi et al., 2014). Additionally, the overexpression of such an 'uncoupled' p120ctn is likely to lead to enough protein localizing in the proximity of the plasma membrane, through simple diffusion in the cytoplasm, even in the absence of any plasma localization signals. Indeed, we know that the overexpression of either N-terminal (aa 1-222) or C-terminal (aa 223-781) of the *Drosophila* p120ctn is sufficient to reduce cellular

elongation in *Drosophila* embryos (Response Figure 2B). This finding is consistent with both fragments retaining a RhoA binding domain, of which there are two mapped along the length of p120ctn (Pieters et al., 2012). Therefore, although the suggested construct would be an interesting tool for the biochemical studies described above, for elucidating the role of p120ctn in cell elongation in the present study we content that the suggested experiment would not produce further insight.




**Response Figure 2. Effects of p120ctn mutations on cell shape**. (**A**) Comparison between human (Query) and *Drosophila* (Sbjct) p120ctn with amino acid residues required for the interaction with E-cad highlighted by red rectangles. (**B**) Effects of expression of different p120ctn variants on cell shape. Note that in this case the p120ctn full-length construct with a different insertion site in *Drosophila* genome was used than the one used in the manuscript. * – $p < 0.1$.

Comment upon the DV border under compression. How does this fit with other studies, and how does it come about?

Most laser ablation experiments do indeed report "positive" recoil velocities when the vertices of the manipulated junction move apart (Sugimura et al., 2016) We have added a short discussion about the source of observed compression and comparison to the Discussion on page 18:

*"We demonstrate that the elongated cell shape is accompanied by anisotropic forces in the epidermis: while the AP borders are under tension, the DV borders are under compression. Most previous laser ablation experiments have reported positive velocities of the initial recoil when the vertices of the manipulated junction move apart (Sugimura et al., 2016). The only exception apart from our work, to our knowledge, is the case of anisotropic tissue stress in the amnioserosa, where a similar negative recoil was observed during germ-band retraction (McCleery et al., 2019). This germ-band retraction drives the elongation of epidermal cells (Gomez et al., 2016; McCleery et al., 2019). We suggest that the anisotropic pushing by the amnioserosa is likely to be the source of observed compression, as also suggested previously (Hirano et al., 2009)."*

We have also added a model with the interpretation of the laser ablation experiments on Figure 8C and its description on page 19 of the revised manuscript.

Page 10 last sentence of final paragraph, why is it said that p120ctn loss results in a decrease of E-cad-GFP at both AP and DV borders (since one would expect reduced endocytosis and thus enhanced E-cad levels)?
And
Page 14, how is it that "The loss of p120ctn resulted in a uniform decrease in the amount of Arf1- GFP at both the AP and DV borders", when the loss of p120ctn also reduced E-cadherin at both the AP and DV borders. One might think that a reduction in Arf1 function/ endocytosis, would expect to correlate with an increase in E-cadherin levels?

As these two points raised by the reviewer are related, we combine our answer to both to more satisfactorily address them. This sentence on Page 10 reports the experimental data. Although indeed this might not seem intuitive, it is consistent with previously published data (Bulgakova and Brown, 2016). Intriguingly, this reduction is observed only when E-cadherin is expressed from its endogenous promoter, but not when a ubiquitous heterologous promoter is used (Figure S1 in Bulgakova and Brown, 2016). In the latter case, E-cad-GFP levels were not affected at either border type by the loss of p120ctn.

This result is indicative of two phenomena: first the presence of p120ctn independent pathways for E-cadherin internalization (Bulgakova and Brown, 2016; Greig and Bulgakova, 2020, in press); second it indicates the potential involvement of p120ctn in transcriptional regulation – which has been well-established in mammalian literature (e.g. Daniel, 2007). The former we have explored in another work (Greig and Bulgakova, 2020, in press), while the latter has yet to be examined and is beyond the scope of the current study.

Bottom of Page 11, Why is E-cad-GFP less dynamic in p120 overexpressing cells when considered in relation to the model in Figure 7, where it would seem that p120ctn overexpression would be expected to enhance endocytosis?

This point is related to the question above: as discussed, p120ctn overexpression leads to the activation of the RhoA signalling cascade which is sufficient to inhibit E-cad endocytosis and Arf1 recruitment to the plasma membrane as we demonstrate using $RhoA^{CA}$ expression. This overcomes the pro-endocytic role of p120ctn through recruitment of Arf1. We have added a clarification in the discussion to address this phenomenon:
*"As a result, both the depletion and overexpression of p120ctn lead to an increase of immobile E-cad at the cell surface: depletion is likely to do so through directly limiting Arf1 recruitment to the plasma membrane, while overexpression does so through elevating RhoA activity, which then inhibits Arf1."*
Further, we have added the results of new experiments where the loss of p120ctn is rescued by $Arf1^{CA}$, whereas its overexpression is rescued by downregulating RhoA using RhoGEF2 RNAi (see new Fig 5-7).

What about p120ctn effects upon Rac? There is little to no discussion of Rac.

The reviewer is correct that Rac GTPase has also been established to act downstream of p120ctn. Indeed, p120ctn was suggested to activate Rac in the cytoplasm through interaction with Vav2 (Peglion and Etienne-Manneville, 2013). This function of p120ctn is independent of the adherens junctions and mostly reported in migrating cells. Therefore, the effects of Rac downstream of p120ctn are not directly relevant to this study which aimed to understand the role of E-cad dynamics in epithelial cell shape. As such, we have decided not to include a discussion about Rac in the context of the current work.

Page 19, Confusing when use the term "stabilization" in isolation (to mean the immobile fraction as discerned by FRAP) since many readers will instead interpret "stabilization" as meaning an increased level of total protein. Better to spell it out and note that you are referring to the immobile fraction.

We agree that the use of "stable" is misleading, though common in the presentation of FRAP data, and corrected this to "the increase of immobile fraction" in the text.

**Reviewer 2**
Advance Summary and Potential Significance to Field:
In this work, Greig and Bulgakova study the interplay between junctional proteins including E-cadherin and associated p120 and Myosin II regulators in the context of epithelial cell shape changes during Drosophila embryonic development. The authors focus on determining junctional aspect ratios of distinct cell borders, localisation of relevant proteins at cell-cell junctions, tension measurements using laser ablations and FRAP measurements under different conditions (overexpression/knockout). The authors conclude that there is a dual function of p120 in regulating distinct cell borders, which is dependent on two distinct GTPase signalling pathways, RhoA and Arf1. This study extends previous observations (Bulgakova 2013, 2016) and is of potential significant interest to readers in the field and beyond for investigators working on tissue morphogenesis, adhesion and actomyosin regulation.

Reviewer 2 Comments for the Author:
However, the authors fail to provide sufficient evidence and clarifications in multiple aspects of this work, which makes their conclusions quite speculative. Following concerns should be

addressed to make the present work publishable in Journal of Cell Science:

Major concerns:
1) The contribution of adhesion and tension in differential regulation of AP and DV cell shape changes needs clarifications and strengthening.
a) The authors delineate proteins and pathway that might regulate cell shape, but how up or downregulation of these components specifically affect AP and DV junctions is not clear from the aspect ratio measurements alone. Similar changes of aspect ratios can be achieved by multiple ways (such as shortening of DV borders with same AP length, or elongation of AP borders with same DV length). Given the different localisation of proteins at different borders, the specific impact on AP/DV junctions should be indicated where cell shape changes were measured.

In all cases for which we observed a reduction of the aspect ratio, we detected a shorter length of the AP borders. However, in cases when increased RhoA activity or MyoII recruitment were detected (e.g. p120ctn overexpression and $RhoA^{CA}$) the shortening of AP borders was accompanied by an increase in cell width's (cell's short axis). This is surprising as increased MyoII accumulation and cortical contractility are expected to act towards the reduction of an intercellular contact length (Lecuit and Lenne, 2007). An explanation we suggest is that the viscoelastic properties of cells in a tissue make them behave according to the Poisson's effect, whereby a material's elongation along one axis tends to result in the material contracting along the transverse axis. This effect is measured using Poisson's ratio: the negative of the ratio of transverse and axial strains. Cells in a tissue demonstrate viscoelastic behaviour rather than elastic (Iyer *et al.*, 2019) and their Poisson's ratio is likely to depend on stress and time (Hilton, 2017). Previous reports which measured a Poisson ratio in cells in the range of 0.35-0.4 (Trickey *et al.*, 2006). Considering this range, it is expected that any change in transverse strain will be proportional to the negative of the product of Poisson ratio and axial strain, and thus the transverse (DV) strain would decrease with increasing axial (AP) strain. At the same time, this effect was not observed (or was beyond the detection limit) in the cases of $Arf1^{DN}$ or $Shibire^{DN}$, as despite changes in junctional dynamics, there was no effect on actomyosin (see Fig. 4 and S3 in the manuscript for $Arf1^{DN}$). To address this question, we have included the mean values of the AP and DV border lengths alongside aspect ratios in the Supplementary Table S1 to indicate where cell shape changes were measured.

b) From the presented images (e.g. Fig. 2A, D), knockout of p120ctn seems to produce more elongated cells (along AP border) compared to control cells. It is surprising that the aspect ratio when quantified did not change (e.g. Fig 2C). As overexpression of p120ctn seems to result in less elongated cells (Fig. 2A, B) it would be logical to expect the opposite effect in the knockout situation. How do the authors explain this discrepancy?

This question was raised by all three reviewers and thus highlights an important point. We refer here to the detailed answer of this question on page 1 of this response.

2) 2 photons lasers ablations has previously successfully been used to determine tension of epithelial junctions in Drosophila, However, the tension measurements presented here seem currently very superficial.
a) Due to the low quality images from the laser cuts it is not clear where the cuts are made and how the junctions/vertices behave after severing. A better representation of the laser ablation experiments such as a kymograph should be included, the dual-colour overlay is very hard to interpret, especially at the DV borders. It would also help to plot distance over time (or speed) to visualise the recoil dynamics.

As the reviewer suggests, we have added plots of distance over time to visualise the recoil dynamics (see new Figures 1 and 2).

b) Further, the accumulation of what looks like a cavitation bubble at the junctions after ablation is visible (Fig. 1G, 2G) and indicates a wound healing response rather than a clean junctional cut. A control for leakage due to introduction of a wound should be included (cytoplasmic fluorophore) to exclude cell membrane damaging.

The "bubble" observed by the reviewer corresponds to the damage to the vitelline membrane – a

rigid envelope outlining embryos and being in close proximity to the ablated epidermal cells. Such discolorations in vitelline membrane are commonly observed in laser ablation experiments in embryos (e.g. Kong et al., 2019; McCleery et al., 2019; Yu and Fernandez-Gonzalez, 2016) and are a fold smaller than cavitation bubbles (e.g. Supatto et al., 2005). At the same time, the cavitation bubble would not necessary be visible using the 488 nm laser (e.g. Colombelli and Solon, 2013). The laser ablation experiments were performed by J.G. under guidance of Dr Tetley in the laboratory of Dr Mao, UCL, where this technique is well-established (e.g. Duda et al., 2019; Mao et al., 2013) and junctional laser ablation is performed routinely.

c) Laser ablation is commonly used to measure line tension in epithelial cells, where recoil indicates outward directed tension before ablation due to force balance of junctional tension at the vertices (e.g. Rauzi and Lenne, Curr Top Dev Biol 2011). How do the authors explain measurements of compression with laser ablation? Compression would probably result in buckling of junctions after ablation, which would make it hard to interpret vertices movements after tension release. This is to my knowledge a new approach and needs explanation.

We did not observe any buckling following ablation of the junctions. Though tissue compression does indeed lead to events such as bending, buckling, and folding in epithelia, the elegant work by Kabla and Charras demonstrated that epithelia can accommodate large and rapid compressive forces extremely well (Wyatt et al., 2020). Additionally, the ability of the embryonic epidermis to “buckle” is abolished by the presence of vitelline membrane, which limits any tissue movement outside of its “z-plane”. This makes the *Drosophila* embryonic epidermis uniquely suited to the study tissue compression *in vivo*. We have added a sentence clarifying the absence of any out of plane movement in the material and methods on page 27.

What model could be used here to describe the ratio measurements of cortical forces? A graphical illustration would help to understand the rationale.

We have now added a discussion about the model on page 18 and a graphical representation in Fig. 8C. This is indeed a new approach, however, as it does not represent the main conclusion of the paper, the development of a detailed mathematical model and further studies of this effect are outside of the scope of this work.

d) For reproducibility, the authors should include the measured time points for each ablation experiment, and in the methods section the physical parameters (pulse duration, power density) and the method used to track the vertices.

We have added plots of distance over time for each genotype and additional information about ablation parameters in the materials and methods section on page 27.

3) The FRAP measurements are a very useful tool to measure dynamics of junctional proteins. However, following aspects need clarifications/considerations:
a) It would be valuable for the reader to show images of the bleached area before/at/after bleaching to visualise where and how much of the junctions were bleached. Also, an indication of the recorded time points measured should be mentioned in the text or/and in the figure legends.

We have added representative images of pre-bleached, immediately after bleaching, and at the end of recovery recording for each FRAP experiment. We do not know what further information the reviewer means by “the recorded time points measured”, as each time point (i.e. for 15 min at 20 sec intervals in case of E-cadherin) was measured and used for plotting the graphs and fitting a model of recovery, the description of which is in the methods section.

b) The authors use the FRAP measurements to exclusively determine mobile/immobile fraction to estimate endocytic recycling. An important component of cell-cell adhesion regulation is dynamics of E-cadherin by diffusion of molecules from/to and within the cell membrane, especially in the context of cortical actomyosin. As the authors already have the FRAP measurements in hand, it would be valuable to determine the half-life of the recovery for the different experiments to consider factors independent of endocytosis.

We are surprized by this comment, as all the best-fit half times are available in the Table S1. We have now submitted a corrected and updated Supplementary Table S1 and would suggest the reviewer refers to this table for all values. Indeed, both our and other labs have demonstrated that E-cadherin does recover through a combination of diffusion and endocytic trafficking (e.g. Bulgakova et al., 2013; Iyer et al., 2019). Additionally, we have previously demonstrated that it is the endocytic trafficking, which is affected by the loss of p120ctn, rather than diffusion (Bulgakova and Brown, 2016). In FRAP experiments, the half-time of recovery driven by diffusion depends on multiple parameters of the experimental set up, including the size of bleach spot, speed of bleaching, temperature, etc (e.g. Kitamura and Kinjo, 2018). In the case of the experiments in the current manuscript, all FRAP curves were best-fit with a single exponential model, and half-times were the same in all datasets (p=0.82). This suggests that with the used setup it was not possible to unmix diffusional and endocytic components due to either close means or high variance.
Increasing the N-number might or might not help the separation of two components, however this will not affect the main conclusion about the immobilization of E-cadherin, especially in the light of observed changes in the dynamics of CLC-GFP as discussed in more details below.

c) The plateaus are not visible in most graphs; the recovery seems still ongoing. To clearly indicate saturation, the plots should be extended to plateau level.

We do appreciate that the recovery is not complete in some of the case. For this reason, we rely on fitting the data rather than using the values of the recovery at the end of the time-series to extrapolate the maximum recovery and the immobile fraction. This is an approach which is well-established in the field, and is commonly used for analysis of long-recovering molecules such as transmembrane proteins (Cohen et al., 2016; Iyer et al., 2019; Sidor et al., 2020; Warrington et al., 2017, and many more). Although increasing the duration of the FRAP experiments might increase the precision of the best-fit values, it will not affect the observed differences in the dynamics.

4) Using CLC FRAP measurements as a proxy for E-cadherin dynamics is an oversimplification, as regulation of E-cadherin endocytosis was shown to occur also in a clathrin-independent way such as through caveolin-mediated internalisation or lipid raft-mediated endocytosis.

There is no caveolin in *Drosophila*, but the reviewer is correct that there are other mechanisms for E-cadherin internalisation. Thus, we have recently found that flotillin is required for the stabilization of immobile E-cadherin (Greig and Bulgakova, 2020, in press) and is likely to be responsible for internalization of the “hour-scale” recovery of E-cadherin (Iyer et al., 2019). However, in this work we were interested specifically in the clathrin-mediated endocytosis of E-cadherin and roles of p120ctn in this process. This interest was motivated by a dramatic change in the dynamics of CLC and its immobilization following both loss and overexpression of p120ctn highlighted in Fig 4B-E.

We have now further validated this approach by comparing CLC-GFP recovery with E-cadherin recovery in cells expressing $RhoA^{CA}$: in both cases we see a similar effect, namely an increase in the immobile fraction. We have added the new analysis of E-cad-GFP FRAP in the presence of $RhoA^{CA}$ to the revised figures (see new Fig. 4) with the CLC-GFP FRAP now placed in Fig. S2 as it supports the conclusions presented in the manuscript.

Therefore, it would make interpretations difficult and might most likely also affect important parameters, the time scale of endocytosis as can be in Fig. 4B, C vs Fig. 3J, K.

Indeed, the recoveries on Fig 4B-C and 3J-K appear to occur at the different time scale. This is exactly when determining the mode of recovery (e.g. single or double exponential) and relative half times become very important as highlighted by the reviewer above. In the case of CLC-GFP the recovery appears faster as there are two components – fast recovery on the scale of seconds (likely through diffusion) and slow recovery on the scale of minutes. The large contribution of the fast component makes the recovery to appear faster than that of E-cadherin. However, surprisingly the slow component recovers on a time scale, which is comparable to E-cad recovery (minutes, see Supplementary Table S1); and it is this slow component whose magnitude is affected most by changes in p120ctn (all best fit data can be found in the Table S1). We agree

that accurate interpretation of these observations would require additional research into mechanisms of CLC dynamics, which is outside of the scope of this work. For this reason, we only used the size of immobile fraction as the clear readout of endocytosis dynamics: an increase of the CLC-GFP immobile fraction is a long-established method to detect inhibition of clathrin-mediated endocytosis. In a proof-of-principle study, reduced CLC-GFP recovery due to an increased immobile fraction was observed in HeLa cells either expressing dominant-negative dynamin or depleted of cholesterol (Wu et al., 2001) We have added this additional justification: *"CLC-GFP incorporates functionally into clathrin-coated pits (Chang et al., 2002; Gaidarov et al., 1999; Kochubey et al., 2006), and its recovery in FRAP reflects endocytic dynamics: immobile fractions of CLC- GFP increased in HeLa cells with downregulated endocytosis (Wu et al., 2001)."*

Also, from Fig. 4B, C it is not clear if AP or DV borders were measured.

Although it was explained in the materials and methods, we have now added a diagram on Figure 4B. In this case, we used a rectangular bleach spot which spans the entire width of the *engrailed-* expressing stripe, and therefore corresponds to combinatorial recovery of CLC-GFP at the AP borders, at which the most changes in protein localization, e.g. $Rok^{KD}$-Venus and MyoII, were observed, and in the cytoplasm in the plane of the adherens junctions. This might not be an ideal solution, but it is robust, reliable, and reproducible as discussed in the answer to the next comment. This, in addition to the fact that the conclusions are the same whether this approach or FRAP of E-cad were used, as demonstrated by comparing the same genotypes (Figures 3, 4, and S2), makes this approach a valid alternative to using E-cad-GFP.

Instead of using this indirect approach, I was wondering why the authors did not continue measuring E-cadherin dynamics directly (as done in Fig. 3), which would give a much better indication of the process.

In addition to the advantages outlined above: the sensitivity and specificity to changes in the clathrin-mediated endocytic pathway, while reflecting the changes in E-cadherin dynamics, this method has another important advantage in comparison to E-cadherin FRAP. Specifically, it can be performed at much higher temporal resolution within a shorter overall time series. This enables automated unbiased quantification of the recovery (the script is available at https://github.com/nbul/FRAP), which is impossible in the case of E-cadherin-GFP FRAP at this developmental stage (even the best time series registration algorithms do not produce a perfectly still movie due to cell shape fluctuations requiring manual correction). Such automated unbiased approaches significantly increases the reproducibility of the data as it is easy to recreate the analysis from the microscope image meta-data, thus helping to overcome the reproducibility crisis in biomedical science (for more detail see Fanelli, 2018; Hunter, 2017). Taken together, these aspects of the CLC-GFP FRAP method makes it a valid, and potentially a better, alternative to studying clathrin-mediated endocytosis of E-cadherin in comparison to E-cadherin FRAP. We have added additional justification of the choice, and clarification of the approach as described above.

5) The link between p120-E-cad-RhoA is intriguing, but needs to be better supported:
a) The authors claim that DN-Rho seems to reduce intensities of E-cad at AP borders. From the images (eg. Fig. 4D) it seems that E-cad localisation changes from continuous decoration along AP borders (control) to spot accumulations, which indicates a reorganisation of E-cad along the junctions, rather than increased endocytosis.

The reviewer is correct that such strong manipulation as the expression of $RhoA^{DN}$ (even 4 hours after induction) has profound effects on E-cad producing a relatively discontinuous accumulation of E-cad along the junctions. Despite this reorganisation, there is evidence of increased E-cad endocytosis, as the total amount of E-cadherin around the cell periphery is reduced (see new Fig. S2). We speculate that the observed spots result from the clustering of remaining E-cadherin, in an opposite process to that of adherens junction formation (reviewed in Green et al., 2010). Given the observation we have with prolonged exposure of the cells to $RhoA^{DN}$, we expect that this remaining E-cadherin will be internalized later as well. Additional evidence for the role of RhoA signalling in the size of the E-cadherin immobile fraction and endocytosis in this system comes from earlier work by N.A.B.: overexpression of RhoGEF2 simultaneously increases E-cadherin levels at the plasma membrane and its immobile fraction, while RhoGEF2

downregulation with RNAi produces the opposite effects (Bulgakova et al., 2013). At the time the authors could not explain the observed phenotypes, however, they make perfect sense in the light of findings of the current manuscript. We have included the following sentence which refers to these data on page 13:
*"This finding was consistent with the increase of both E-cad levels and immobile fraction in cells overexpressing the RhoA activator RhoGEF2, and the opposite effect upon its downregulation (Bulgakova et al., 2013)."*

b) The authors explore a possible link between E-cad and RhoA in Fig. 4, but their conclusions are related to the role of p120 in Rho signalling ("In cells overexpressing p120ctn, the increase of E- cad at AP borders was accompanied by increased activation of RhoA"). In fact, there is not a single experiment that shows what happens to p120 under DN-Rho and ca-Rho conditions and vice versa and the conclusion remains speculative.

As the antibody against p120ctn does not stain reliably in our system (see Figure S2), we could not perform this analysis using endogenous p120ctn. We planned to examine the effect of $RhoA^{CA}$ and $RhoA^{DN}$ on *Ubi*::p120ctn-GFP. At the time of arranging these experiments the country was placed in lockdown due to the ongoing coronavirus outbreak and we were unable to complete these experiments. However, we would expect p120ctn to be affected in a similar fashion to E-cad, as p120ctn displays the same localization and asymmetry between borders as E-cad. These results might have provided some additional support for our findings, but are unlikely to add new insights.

The authors mention that p120 has a tension-dependent function, but direct evidence is missing.

It is unclear what the reviewer means by "direct evidence". We only mention p120ctn "tension-dependent" function as a possible explanation for the observed differences in the effects at AP and DV borders. The feasibility of this explanation is supported by the recently demonstrated tension- dependent function of p120ctn in another *Drosophila* epithelium (Iyer et al., 2019). Providing a biophysical proof of this function through, for example single-molecule experiments, is outside of the scope of this work.

c) From the FRAP measurements in Fig. 4I it is not clear if DV or AP borders were measured. Is there any change in dynamics in the case of DN-Rho?

We have clarified the FRAP measurements in Fig. 4I above and added a diagram of how the experiments were performed. Performing FRAP in cells expressing $RhoA^{DN}$ was not possible: the constant expression of $RhoA^{DN}$ abolishes E-cadherin localization to the plasma membrane, whereas an intermediate state following acute induction of its expression does not yield a steady state (as we know that all E-cadherin is internalized at the end point) making the resulting FRAP curve non-interpretable. However, a mild downregulation of RhoA signalling with RhoGEF2-RNAi resulted in increased E-cadherin turnover (Bulgakova et al., 2013), expectedly and oppositely of what is observed in cells expressing $RhoA^{CA}$ or overexpressing RhoGEF2.

d) As mentioned in point 4 above, instead of measuring CLC dynamics (Fig. 4), FRAP on E-cad should be performed, which would give a direct readout for E-cad dynamics and would clearly indicate how elevated and reduced levels of RhoA control E-cad turnover at AP/DV borders.

As described above we have now performed and incorporated data about E-cadherin turnover in cells expressing $RhoA^{CA}$. We have additionally added references and discussion about previous findings about how the modulation of RhoGEF2 expression affect E-cad levels and turnover. These findings support our conclusions from the measuring of CLC dynamics.

6) The authors mention that RhoA prevents the localisation of Arf1 at the plasma membrane (Fig. 7B), but no direct evidence is shown. For example, what happens to Arf1 in ca-Rho expressing cells?

We have examined Arf1-GFP localization in $RhoA^{CA}$ expressing cells and found a reduction at the cell surface ($p=0.025$) This data supports our finding about the effects of RhoA signalling on Arf1-GFP obtained using RNAi against RhoGEF2 and was added to the revised Figure 6.

7) Fig. 4F and Fig. 6C seem identical.

The figures to which the reviewer refers are indeed identical images to highlight that these findings refer to two different aspects – E-cadherin levels and cell shape – of the same dataset with same genotypes/manipulation. Such use of image duplication is not considered inappropriate or problematic (Bik et al., 2016).

Minor points:
1) As the authors indicate actomyosin as major regulator, it would be valuable to investigate if also actin localisation is anisotropic (like Myo II).

We have measured actin localization in the embryonic epidermis at this stage using LifeAct-GFP as a marker, and found that it is isotropic and unaffected by the overexpression of p120ctn. We have added these data into Supplementary Figure S1.

2) The cell shape change called “cell rounding” is misleading and should be changed, as this terminology is commonly used for cells rounding up due to mitosis. The presented cells in the epithelia after manipulations appear at best less elongated (e.g. Fig. 2A), but not round.

We agree and replaced it with “reduced elongation” throughout the text.

3) The graphs are often not aligned with the images, which makes it difficult to find the corresponding graph amongst all the panels within the figures. This should be adjusted.

We have adjusted and aligned graphs where permissible by space constrains.

4) Fig. 3D: The E-cad levels at the DV borders show a very high standard deviation compared to AP and need more data points to clearly demonstrate no significance.

We agree with the reviewer that the figure showed a slightly higher than usual SD. We have repeated this experiment to confirm the finding and have included this with the revised manuscript.

3) Fig. 5F: It seems as there are 3 curves plotted, I believe there should be only 2.

We thank the reviewer for spotting this error and we have corrected it.

**Reviewer 3**
This manuscript by Bulgakova and colleagues examines the interplay between adhesion and contractility in the regulation of cell shape in the late Drosophila embryonic epidermis. Using genetic perturbations that modulate adhesion dynamics and/or actomyosin contractility, they argue that the acquisition of the anisotropic and elongated cell shape is the result of the “counteracting” effects of actomyosin contractility that suppresses cell elongation, and adhesion that promotes it. They arrive at these conclusions through the analysis of cell shape, contractility and adhesion dynamics in regulators of either or both. Specifically, they examine the effects of altering the levels p120catenin- a known regulator of adhesion dynamics, Rho GTPase signaling which influences both adhesion and contractility, and the Arf1 GTPase that they show here influences adhesion dynamics downstream of p120 catenin. They propose a model in which they suggest that at the long interfaces of epidermal cells, p120 catenin activates both Rho and Arf GTPases to respectively regulate contractility and adhesion dynamics, with Rho activation also inhibiting Arf1 GTPase. That contact expansion requires the downregulation of contractility has been established in many systems. It has also been demonstrated that in elongated cells with anisotropic shapes, the distribution of myosin and ECadherin/Bazooka are complementary. What molecular mechanisms mediate these distribution patterns, how they may influence one another and whether these patterns govern the acquisition of anisotropic shapes are challenging questions that this manuscript addresses, using p120catenin (which one of the authors had previously demonstrated modulates ECadherin dynamics by specifically affecting the mobile ECadherin pool that is in a complex with Bazooka) as its center-

point.

Although the work presented in this manuscript demonstrates the reciprocal and anisotropic effects of upregulating or downregulating p120 levels on actomyosin contractility and ECadherin levels, their effects on Ecadherin dynamics (increase and decrease respectively), the effects on clathrin dynamics and Arf GTPase distribution in the two perturbations are similar. Also, while p120 overexpression leads to a reduced anisotropy in cell shape, loss of p120 function has no effect on shape. To substantiate the model they propose, the authors examine the consequences of reduced or increased contractility (using Rho/RhoGEF2 knock down/activation) and identify effects on Ecadherin distribution and clathrin dynamics. Since most of the conclusions made are based on the similarities in the phenotypes of different classes of perturbations, some of the statements made (eg: ECadherin was also stabilized when Rho signaling was downregulated in p120 mutants, p14; and we have demonstrated that p120 regulates actomyosin dynamics via RhoA signaling and ECadherin dynamics via both RhoA and Arf1; p16) are too strong and must be validated by genetic rescue experiments. In the only rescue experiment presented in this paper, the authors show that activating Arf1 in a p120 mutant restores clathrin dynamics to wildtype levels. While this illustrates a role for Arf1 in regulating Ecadherin dynamics, the influence of p120 dependent Ecadherin dynamics on cell shape anisotropy remains unclear. Also, while the results demonstrate that cell shape is influenced by regulators of adhesion and contractility, they also reveal that the picture is complex. In their present form, the experiments do not provide a clear mechanistic picture. Overall, I think the novelty of this paper resides in the demonstration of the requirement of Arf in regulation of anisotropic shape (Fig.S4), its modulation of clathrin dynamics, and the (similar) modulation of its levels by both p120 overexpression and knockdown. I am of the opinion that the paper will benefit from validating one or both arms of the proposed model through rescue experiments that examine the effects of specifically modulating either Ecadherin dynamics or contractility on cell shape changes induced by p120 overexpression or Arf1 knockdown or identifying why overexpression rather than knockdown of p120 influences cell shape. Might p120 levels under tight regulation?

We thank the reviewer for highlighting that our manuscript is of interest and we agree that the discovery of a role for Arf1 is one of the key findings of our study. Although the reviewer does not specify which rescue experiments they have in mind, we did include a rescue of CLC dynamics by Arf1 in a p120ctn mutant genetic background in the original manuscript (Fig. 5). To strengthen our arguments, we have now added two rescue experiments related to two different aspects of the proposed mechanism, which we are confident validate and strengthen our evidence. Specifically, we found that:

a) Supressing RhoA signalling using RhoGEF2-RNAi in cells overexpressing p120ctn rescues the cell elongation defect and increases Arf1 localization to the cell surface (see new Fig. 5, 6, 7)

b) Elevating Arf1 activity using $Arf1^{CA}$ in a *p120ctn* mutant background rescues the cell elongation phenotype, newly identified when carefully analysing the *engrailed*-positive compartment (see page 1 of this response). This replicates and complements the previous experiments showing the rescue of CLC-GFP dynamics in this genotype (see new Figures 5 and 7).

Together these experiments add further support to the model we propose, and we are content that this addresses the additional investigations requested by the reviewer.

Other concerns:
In Fig.1, the images do not clearly show the effects of ablation. It would desirable to show a time series.

We have added a time series to Figure 1.

In p120 loss of function mutants, were embryos examined for phenotypes in the engrailed domain only? How do the authors explain the lack of effect of p120 reduction on cell shape?

This question was raised by all three reviewers and thus highlights an important point. We refer here to the detailed answer of this question on page 1 of this response.

In Fig.5, single images would be helpful to visualize effects on the membrane distribution of Arf1. In the projections, no membrane localization is evident.

Although the single plane images are appealing for illustrative purposes, it is the projections which reflect the change in Arf1 localization and amounts and were used for comparing the genotypes as demonstrated on graphs. Therefore, we are confident that it is much more important to show these projections rather than visually pleasant but scientifically meaningless single sections, while showing both would overcrowd the figures. To highlight this point we have now places the single plane image in the Supplementary Figure S3 as it does not add new information.

It would be useful to have a table summarizing the effects of various perturbations tested on the parameters analysed.

We have added a summarizing table on Figure 8 as suggested by the reviewer.

The legend to Supplementary Figure 4 does not correspond with the figure supplied. Some panels are missing.

We have incorporated some of the data from Figure S4 into the revised Figure S3 and removed some of the analysis originally presented in Figure S4 as it does not provide additional insight. Therefore, the revised manuscript has 3 supplementary figures rather than the 4 included with the original manuscript. Nonetheless, we thank the reviewer for identifying this point and have taken additional measures to ensure that the legends provided with the revised manuscript are accurate.

**References:**

Bik, E.M., A. Casadevall, and F.C. Fang. 2016. The Prevalence of Inappropriate Image Duplication in Biomedical Research Publications. *mBio*. 7.
Brigidi, G.S., Y. Sun, D. Beccano-Kelly, K. Pitman, M. Mobasser, S.L. Borgland, A.J. Milnerwood, and
S.X. Bamji. 2014. Palmitoylation of delta-catenin by DHHC5 mediates activity-induced synapse plasticity. *Nat Neurosci*. 17:522-532.
Bulgakova, N.A., and N.H. Brown. 2016. Drosophila p120-catenin is crucial for endocytosis of the dynamic E-cadherin-Bazooka complex. *Journal of Cell Science*. 129:477-482.
Bulgakova, N.A., I. Grigoriev, A.S. Yap, A. Akhmanova, and N.H. Brown. 2013. Dynamic microtubules produce an asymmetric E-cadherin-Bazooka complex to maintain segment boundaries. *J Cell Biol*. 201:887-901.
Chang, H.C., S.L. Newmyer, M.J. Hull, M. Ebersold, S.L. Schmid, and I. Mellman. 2002. Hsc70 is required for endocytosis and clathrin function in Drosophila. *J Cell Biol*. 159:477-487.
Cohen, D.J., M. Gloerich, and W.J. Nelson. 2016. Epithelial self-healing is recapitulated by a 3D biomimetic E-cadherin junction. *Proc Natl Acad Sci U S A*. 113:14698-14703.
Colombelli, J., and J. Solon. 2013. Force communication in multicellular tissues addressed by laser nanosurgery. *Cell Tissue Res*. 352:133-147.
Daniel, J.M. 2007. Dancing in and out of the nucleus: p120(ctn) and the transcription factor Kaiso.
*Biochim Biophys Acta*. 1773:59-68.
Duda, M., N.J. Kirkland, N. Khalilgharibi, M. Tozluoglu, A.C. Yuen, N. Carpi, A. Bove, M. Piel, G. Charras, B. Baum, and Y. Mao. 2019. Polarization of Myosin II Refines Tissue Material Properties to Buffer Mechanical Stress. *Dev Cell*. 48:245-260 e247.
Fanelli, D. 2018. Opinion: Is science really facing a reproducibility crisis, and do we need it to? *Proc Natl Acad Sci U S A*. 115:2628-2631.
Gaidarov, I., F. Santini, R.A. Warren, and J.H. Keen. 1999. Spatial control of coated-pit dynamics in living cells. *Nature Cell Biology*. 1:1-7.
Gomez, J.M., L. Chumakova, N.A. Bulgakova, and N.H. Brown. 2016. Microtubule organization is determined by the shape of epithelial cells. *Nat Commun*. 7:13172.
Green, K.J., S. Getsios, S. Troyanovsky, and L.M. Godsel. 2010. Intercellular junction assembly, dynamics, and homeostasis. *Cold Spring Harb Perspect Biol*. 2:a000125.
Greig, J., and N.A. Bulgakova. 2020. Arf6 determines tissue architecture by stabilizing intercellular adhesion. *Phil Trans R Soc Lond B Biol Sci*. in press. doi: 10.1098/rstb.2019.0682

Hirano, M., D. Neff, and S. Collier. 2009. Cell shape and epithelial patterning in the Drosophila embryonic epidermis. *Fly (Austin)*. 3:185-191.
Hunter, P. 2017. The reproducibility "crisis": Reaction to replication crisis should not stifle innovation. *EMBO Rep*. 18:1493-1496.
Ishiyama, N., S.H. Lee, S. Liu, G.Y. Li, M.J. Smith, L.F. Reichardt, and M. Ikura. 2010. Dynamic and static interactions between p120 catenin and E-cadherin regulate the stability of cell-cell adhesion. *Cell*. 141:117-128.
Iyer, K.V., R. Piscitello-Gomez, J. Paijmans, F. Julicher, and S. Eaton. 2019. Epithelial Viscoelasticity Is Regulated by Mechanosensitive E-cadherin Turnover. *Curr Biol*. 29:578-+.
Kitamura, A., and M. Kinjo. 2018. Determination of diffusion coefficients in live cells using fluorescence recovery after photobleaching with wide-field fluorescence microscopy. *Biophys Physicobiol*. 15:1-7.
Kochubey, O., A. Majumdar, and J. Klingauf. 2006. Imaging clathrin dynamics in Drosophila melanogaster hemocytes reveals a role for actin in vesicle fission. *Traffic*. 7:1614-1627.
Kong, D., Z. Lv, M. Haring, B. Lin, F. Wolf, and J. Grosshans. 2019. In vivo optochemical control of cell contractility at single-cell resolution. *EMBO Rep*. 20:e47755.
Lecuit, T., and P.F. Lenne. 2007. Cell surface mechanics and the control of cell shape, tissue patterns and morphogenesis. *Nat Rev Mol Cell Biol*. 8:633-644.
Mao, Y.L., A.L. Tournier, A. Hoppe, L. Kester, B.J. Thompson, and N. Tapon. 2013. Differential proliferation rates generate patterns of mechanical tension that orient tissue growth. *Embo J*. 32:2790-2803.
McCleery, W.T., J. Veldhuis, M.E. Bennett, H.E. Lynch, X. Ma, G.W. Brodland, and M.S. Hutson. 2019. Elongated Cells Drive Morphogenesis in a Surface-Wrapped Finite-Element Model of Germband Retraction. *Biophys J*. 117:157-169.
Munjal, A., J.M. Philippe, E. Munro, and T. Lecuit. 2015. A self-organized biomechanical network drives shape changes during tissue morphogenesis. *Nature*. 524:351-355.
Peglion, F., and S. Etienne-Manneville. 2013. p120catenin alteration in cancer and its role in tumour invasion. *Phil Trans R Soc Lond B Biol Sci*. 368:20130015.
Pieters, T., F. van Roy, and J. van Hengel. 2012. Functions of p120ctn isoforms in cell-cell adhesion and intracellular signaling. *Front Biosci (Landmark Ed)*. 17:1669-1694.
Sidor, C., T.J. Stevens, L. Jin, J. Boulanger, and K. Roper. 2020. Rho-Kinase Planar Polarization at Tissue Boundaries Depends on Phospho-regulation of Membrane Residence Time. *Dev Cell*. 52:364-378 e367.
Sugimura, K., P.F. Lenne, and F. Graner. 2016. Measuring forces and stresses in situ in living tissues. *Development*. 143:186-196.
Supatto, W., D. Debarre, B. Moulia, E. Brouzes, J.L. Martin, E. Farge, and E. Beaurepaire. 2005. In vivo modulation of morphogenetic movements in Drosophila embryos with femtosecond laser pulses. *Proc Natl Acad Sci U S A*. 102:1047-1052.
Warrington, S.J., H. Strutt, K.H. Fisher, and D. Strutt. 2017. A Dual Function for Prickle in Regulating Frizzled Stability during Feedback-Dependent Amplification of Planar Polarity. *Curr Biol*.
27:2784-2797 e2783.
Wu, X., X. Zhao, L. Baylor, S. Kaushal, E. Eisenberg, and L.E. Greene. 2001. Clathrin exchange during clathrin-mediated endocytosis. *J Cell Biol*. 155:291-300.
Wyatt, T.P.J., J. Fouchard, A. Lisica, N. Khalilgharibi, B. Baum, P. Recho, A.J. Kabla, and G.T. Charras.
2020. Actomyosin controls planarity and folding of epithelia in response to compression.
*Nat Mater*. 19:109-117.
Yu, J.C., and R. Fernandez-Gonzalez. 2016. Local mechanical forces promote polarized junctional assembly and axis elongation in Drosophila. *Elife*. 5.

---

Second decision letter

MS ID#: JOCES/2019/242321

MS TITLE: Interplay between actomyosin and E-cadherin dynamics regulates cell shape in the Drosophila embryonic epidermis.

AUTHORS: Joshua Greig and Natalia A Bulgakova
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favorable reports but one referee raised some critical points that will require amendments to your manuscript. Please do the best you can to address these remaining comments and then I will make a final decision at the editorial level.

*We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.*

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

Please see advances/ significance as mentioned in prior review.

*Comments for the author*

The authors have responded satisfactorily/ well to this reviewer's concerns.

Reviewer 2

*Advance summary and potential significance to field*

The submitted revision is a clearly improved version of the previous manuscript and I believe this study will be of significant interest to readers across different fields including researchers working on tissue development and morphogenesis, actomyosin and cell-cell adhesion and signalling.

*Comments for the author*

This is a much improved version of the initial manuscript. My concerns have been addressed satisfactorily by providing additional evidence, or/and sufficient explanations. I believe this paper will be a valuable addition to JCS.

Reviewer 3

*Advance summary and potential significance to field*

This is a revised manuscript by Bulgakova and colleagues that examines the interplay between adhesion and contractility in the regulation of cell shape in the late Drosophila embryonic epidermis. Using genetic perturbations they demonstrate that the p120 catenin modulates adhesion dynamics and actomyosin contractility through the regulation of the Rho1 and Arf 1 GTPases, and argue that the acquisition of the anisotropic and elongated cell shape is the result of the "counteracting" effects of actomyosin contractility that suppresses cell elongation, and adhesion that promotes it.

To support their claims, they demonstrate that ECadherin and active myosin exhibit mutually exclusive patterns of distribution along the short/DV and long/AP cell edges and show that while p120 distribution mirrors Ecadherin and Rho activity mirrors myosin, the distribution of Arf 1 is isotropic. They analyse the effects on cell shape, contractility and adhesion dynamics upon modulating the levels of p120catenin, the Rho1 and Arf1 GTPases singly and in combination. They use their results to propose that at the long interfaces of epidermal cells, p120 catenin activates both Rho and Arf GTPases to respectively regulate contractility and adhesion dynamics, with Rho activation also inhibiting Arf1 GTPase.

Although the work presented in this manuscript demonstrates the reciprocal and anisotropic effects of upregulating or downregulating p120 levels on actomyosin contractility and ECadherin levels, their effects on Ecadherin dynamics (increase and decrease respectively), the effects on clathrin dynamics and Arf GTPase distribution and cell shape on the two perturbations are similar. They identify a previously missed phenotype in teh p120 catenin mutant that was pointed out by all three reviewers. They also find that increasing contractility (using Rho activation) or knocking down Arf have similar effects on cell shape and that the former reduces Arf1.
They then show in new experiments that were suggested in my previous review of the manuscript, that the effects on cell shape of p120 mutants can be modulated by altering by Rho and Arf. Also, activating Arf1 in a p120 mutant restores clathrin (used as a proxy for ECadherin endocytosis) dynamics to wildtype levels. They suggest based on the similar effects of p120 kd, Rho CA, Arf DN and Shi DN on increasing immobile Ecadherin, a central role for ECadherin dynamics in cell elongation. They use the stronger effect they see in RhoCA to argue that contractility also plays a role.

*Comments for the author*

The manuscript is an improved version of the original and addresses many of my concerns. It identifies a role for p120catenin in modulating adhesion dynamics and contractility through its effects on the Rho1 and Arf1GTPases and identifies a network of interactions between the three. While a role for p120 in regulating adhesion dynamics is clear, whether its regulation of Rho suggests a role for contractility remains unclear (see comment below). I feel a more apt/specific title for the manuscript might be " p120 catenin influences anisotropic cell shape through its effects of Rho1 and Arf1 GTPases". I have a few remaining concerns that I list below.

i) While the rescue of cell shape changes in p120 mutant by Arf CA overexpression are validated, it will be important to ensure that the combination of two UAS lines in the p120 oe, Rho GEF2RNAi combination is not diluting the effects of either RNAi. It will also be useful to determine whether clathrin dynamics is also rescued in this combination.

ii) I find the lack of effects with RhoGEF2RNAi or RhoDN surprising. Could the authors comment on whether downregulating contractility more downstream (eg: myosin phosphatase oe, sqhAA, zip DN) modulates the p120 oe cell shape phenotype?

iii) The effects of ablation on the movement of the vertices and of the morphology of the adjacent cells are still not clearly visible in the images. I feel that the force balance descriptions need additional validation by experiments and that the physical model is does not add much to the main subject of the paper.

Other concerns:
The legends to Figs S1 and S3 have been swapped.

---

**Second revision**

Author response to reviewers' comments

**Answer to reviewer 3.**
The manuscript is an improved version of the original and addresses many of my concerns. It identifies a role for p120catenin in modulating adhesion dynamics and contractility through its effects on the Rho1 and Arf1GTPases and identifies a network of interactions between the three. While a role for p120 in regulating adhesion dynamics is clear, whether its regulation of Rho suggests a role for contractility remains unclear (see comment below). I feel a more apt/specific title for the manuscript might be " p120 catenin influences anisotropic cell shape through its effects of Rho1 and Arf1 GTPases". I have a few remaining concerns that I list below.

We would like to thank the reviewer for their comments and agree that we did address all their previous comments (see also the response to the comment below). We are however reluctant to rename the manuscript – an aspect which did not raise any concerns from other two reviewers. We do indeed use manipulations of p120ctn to simultaneously modulate both E-cadherin dynamics and cortical actomyosin. Nonetheless, it is the interplay between these two processes in cell shape regulation which is the focus of our work in this manuscript, while p120ctn serves as a molecular tool by which we study these mechanisms.

i) While the rescue of cell shape changes in p120 mutant by Arf CA overexpression are validated, it will be important to ensure that the combination of two UAS lines in the p120 oe, Rho GEF2RNAi combination is not diluting the effects of either RNAi. It will also be useful to determine whether clathrin dynamics is also rescued in this combination.

The reviewer highlights an important consideration when performing experiments using the UAS-Gal4 system in *Drosophila*, which we have also indicated on page 8. All our experiments used additional copies of *UAS*::CD8-Cherry, when necessary, to balance the Gal4:UAS ratio across the genotypes in each dataset. For example, in the specific case indicated by the reviewer we used the progeny of the following crosses: ♀ *en*::GAL4 *shg*::E- cad-GFP/ CyO, *twi*::GAL4, *UAS*::GFP; *UAS*::CD8-Cherry/TM6, *dfd*::YFP crossed to: ♂ *UAS*::p120ctn, *UAS*::CD8-Cherry/ TM6, *dfd*::YFP or ♂ *UAS*::RhoGEF2RNAi/ CyO, *dfd*::GFP; *UAS*::p120ctn /TM6, *dfd*::YFP to produce embryos expressing *shg*::E-cad-GFP with either simultaneous overexpression of p120ctn and two copies of CD8-Cherry or simultaneous overexpression of p120ctn and RhoGEF2-RNAi with one copy of CD8-Cherry. Thus, the Gal4:UAS ratio was (1:3) in all embryos, which controls for any Gal4 dilution effect.

Due to the word limit and to avoid overcomplication we could not include such a detailed description of each cross, however we have added a clarifying sentence in materials and methods on page 21: "In all experiments when necessary, additional copies of *UAS*::CD8-Cherry were used to balance the Gal4:UAS ratio across genotypes in each dataset."

While testing if RhoGEF2-RNAi rescues the defects in clathrin dynamics observed following p120ctn overexpression would provide yet one more validation to our model, it will not add novel information about the mechanism. As experiments are not possible in the current situation (see below), we do not consider such validation as being essential.

ii) I find the lack of effects with RhoGEF2RNAi or RhoDN surprising. Could the authors comment on whether downregulating contractility more downstream (eg: myosin phosphatase oe, sqhAA, zip DN) modulates the p120 oe cell shape phenotype?

Although we understand the reviewer's surprise at the lack of effects in these cases, we are

confident in these results as these are two independent approaches to downregulate contractility which have been validated in the past by our lab and by others independently. Considering that downregulating RhoA signalling/contractility leads to elevated E-cad dynamics in both fly and mammalian cells (e.g. Bulgakova et al., 2013, Ratheesh at al., 2012), these results are consistent with the importance of the reported interplay between E-cad dynamics and actomyosin in the regulation of cell shape. When taken together with the effect of activating RhoA, the lack of effects observed with both RhoGEF2-RNAi and $Rho^{DN}$ only strengthen the evidence for the role of contractility.

Using additional approaches to downregulate contractility, as the reviewer suggests, is therefore likely to confirm these results without adding to the mechanism. As we demonstrated that several of the molecules used to explore actomyosin contractility produced the same output (see Fig. 2 and S1), the suggested tools are likely to yield the same result as reported for the two approaches presented in the manuscript. For example, expression of *UAS*::$Zipper^{DN}$ did not produce any effect on cell shape (data not shown). Due to our concerns about the functionality of this published dominant-negative variant we opted not including these data into the manuscript without further validation.

In normal circumstances we could proceed with any additonal validation and exploring additional reagents suggested by the reviewer. However, in the current situation this is not possible: the lab of Dr Bulgakova is currently closed due to the Covid-19 lockdown, while Dr Bulgakova will be on maternity leave from August 2020 and Dr Greig has since left the lab to start his new job in a non-*Drosophila* laboratory.

iii) The effects of ablation on the movement of the vertices and of the morphology of the adjacent cells are still not clearly visible in the images. I feel that the force balance descriptions need additional validation by experiments and that the physical model is does not add much to the main subject of the paper.

Although the other two reviewers had no concerns about the movement of the vertices, we have decided to include four Supplementary Movies with representative ablation experiments for each condition (new Movies S1-4). We hope these movies help to visualise “the movement of the vertices and of the morphology of the adjacent cells” and support the model presented in Figure 8.

We agree with the reviewer that “the physical model does not add much to the main subject of the paper”, therefore we do not see the need for “additional validation by experiments” within scopes of this manuscript. However, we are convinced that these findings are of significant interest to readers across different fields (as indicated by reviewer 2) and are a compelling addition to the rest of the manuscript. Validation of the reported force balance description both experimentally and through novel modelling approaches is a fascinating topic for future research and the subject of a newly established collaboration in the lab.

Other concerns:
The legends to Figs S1 and S3 have been swapped.

We thank the reviewer for pointing this out and corrected the supplementary information file.

---

Third decision letter

MS ID#: JOCES/2019/242321

MS TITLE: Interplay between actomyosin and E-cadherin dynamics regulates cell shape in the Drosophila embryonic epidermis.

AUTHORS: Joshua Greig and Natalia A Bulgakova
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.