nature portfolio

Peer Review File

Nuclear position controls the activity of cortical actomyosin networks powering simultaneous morphogenetic events

Corresponding Author: Dr Matteo Rauzi

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the paper "Nuclear Position Controls the Activity of Cortical Actomyosin Networks Powering Simultaneous Morphogenetic Events," the authors investigate the spatiotemporal regulation of actomyosin required for concomitant tissue folding and cell intercalation, two critical morphological processes. They demonstrate that at the onset of gastrulation, mesoderm cells undergo coordinated apical constriction, generating a basally directed cytoplasmic flow that moves the nuclei towards the cell's basal side. This nuclear translocation triggers the remodeling of the microtubule (MT) network, causing MT-plus ends to shift from the apical cortex to the lateral cortex, thereby redirecting the delivery of RhoGEF2 to the lateral cortex. Thus, the spatiotemporal distribution of MT-plus ends governs the localization of RhoGEF2, which in turn regulates actomyosin localization.

Overall, the authors have addressed most of the immediate questions experimentally, and the manuscript is well-written. Additionally, the methodology used in the paper is of a high standard. However, some results remain a bit hard to interpret at this stage, and additional controls would be needed.

Major Comments

1. Molecular Link Between Nuclear Movement and Microtubules
The novelty of the paper lies in linking microtubule redistribution necessary for lateral RhoGEF2 recruitment to nuclear migration. However, the authors do not provide the molecular link between nuclear movement/localization and microtubules. An alternative explanation could be that microtubules interact with adherens junctions. Apical constriction, together with cytoplasmic flow, might lead to a basal shift of the junctions, which would relocate the MT-plus ends toward lateral sites. This would mean that redistribution of junctions leads to MT-plus ends relocalization. The inhibition of nuclear migration could affect the cytoplasmic flow that accelerates this shift and, thus, indirectly affects cell polarity. I would suggest monitoring the location of adherens junctions (e.g., DE-cadherin) during apical constriction and correlating it with the localization of RhoGEF2 and MT plus ends. Also, it would be interesting to see the effect of blocking nuclear migration on junctions.

2. RhoGEF2 Activation and EB1 Polarity
RhoGEF2 gets activated in very specific foci at the lateral site. Although more MT-plus ends interact with the lateral sides upon nuclear translocation, there is no polarity of EB1 movement before RhoGEF2 localization to the intercalating D-V junction. The authors show in Supp. Fig 10, e, f the similar localization of EB1, but this is observed only after the shrinkage of the intercalating D-V junction. Shouldn't EB1 density precede RhoGEF2 on this junction? It would be beneficial to image EB1 (GFP) together with RhoGEF2 or at least with MyoII (Cherry) to correlate the localization of both. The current results suggest additional molecular players regulate RhoGEF2. Also, it would be insightful to see the formation of lateral MyoII clusters using genetic perturbation of microtubules. For example, removing centrosomes by knocking down sas-4 or asl could provide a clearer picture of microtubule function compared to the complete removal of microtubules by colcemid.

Conclusion

With these additions, I would be very supportive of the publication. The manuscript is convincing overall, but addressing the points above would strengthen the conclusions and provide a more comprehensive understanding of the mechanisms

involved.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This well-written and elegant manuscript presents significant and timely research aimed at explaining how tissues undergo multiple concomitant shape changes during mesoderm folding and extension in the early Drosophila embryo morphogenesis. Previous studies demonstrate that this process requires specifically timed reorganization of cortical myosin in two separate tiers. The first tier of actomyosin localizes to the apical cell cortex and drives apical constriction of the cells, while the second tier localizes to the lateral cell cortex and drives cell intercalation for simultaneous tissue folding and convergence-extension. In this study, the authors present evidence that the nucleus acts as a barrier to shield the lateral cortex from the microtubule network so that the recruitment of RhoGEF2, a key activator of actomyosin, to the lateral cortex is regulated both spatially and temporally. By manipulation of the nucleus in gastrulating embryos, the authors demonstrate that the accumulation and position of lateral actomyosin is tightly associated with the apical-basal position of the nucleus. Furthermore, by combining pharmacological and optogenetic manipulations with computer modeling, the authors propose a model where the cytoplasmic flow caused by apical constriction passively relocates the nucleus and unshields the cells' lateral cortex for RhoGEF2 delivery by microtubules (MT), in order to designate the formation of the second actomyosin tier. Finally, the authors show evidence that this mechanism requires the coordinated function of the MT plus-end binding protein EB1 and the transmembrane protein T48, a developmentally regulated cortical anchor for RhoGEF2.

This study provides a new perspective on the nucleus as a spatiotemporal cytoskeleton compartmentalizer that drives part of the morphogenic process mediating coordinated epithelial folding and tissue extension. These results are important and worthy of publication as they offer a more holistic view of morphogenesis by deciphering how tissues undergo various simultaneous shape changes during morphogenesis and how cellular organelles can change position to provide spatial regulation of the force-generating machinery in the cell. The authors employed multiple approaches to test their main conclusions, including computer modeling, which is a significant strength of the paper. The data are well-quantified and thoroughly analyzed. However, for certain methods used in the study, additional control experiments are necessary to accurately assess the actual effects of the treatment. These controls would strengthen the validity of the conclusions and provide a clearer understanding of the treatment's impact.

Major points

1. In the experiments where the authors used laser ablation to dissect the apical actomyosin network (illustrated in Figure 2a and 2b), the apical area of the cells was greatly dilated after laser dissection, causing profound changes in cell shape. The authors should rule out the possibility that the reduction of lateral myosin intensity is due to dilution of the cortical signal as the cells were stretched, or due to a shift of myosin enrichment to a more apical location on the lateral cortex. In addition, to rule out a potential direct impact of laser ablation on lateral myosin, in particular in those experiments where laser ablation was iteratively applied to the embryos, the authors should show evidence that the lateral myosin is still present immediately after the laser treatment but disappears over time as the nuclei migrate apically. Finally, in the laser-dissected region, it appears that there were still some nuclei localized at a basal position comparable to some nuclei in the uncut control region (as illustrated by the partially overlapped distributions of alex-to-nucleus distance shown in Fig. 2d). Could lateral myosin be detected in the vicinity of these more basally localized nuclei in the dissected region?

2. The evidence supporting the halfway rule (model 3) for lateral myosin localization should be strengthened further, or the conclusion should be softened. In Fig. 3b, the standard deviation for model 3 is not markedly different from model 2, where the position of lateral myosin is determined by the most basal nuclei of the four. Was the difference between the two models statistically significant? Additionally, the observations presented in the remaining parts of Fig. 3 (Fig. 3c – g) did not provide further distinction among the proposed models. It might be helpful to examine conditions where the neighboring nuclei have more distinct apical-basal locations to further test the halfway rule. For example, the author could examine the cells located at the border of the cut and uncut zones. Alternatively, the authors could examine nuclei in charleston (char) mutants, where there are irregularities in the alignment of the nuclei (Char is a regulator of nuclear morphogenesis and apical nuclear anchoring, and its RNAi embryo has wildtype microtubule organization and association to the nuclear envelope. PMID: 16421189). An interesting question (which might be beyond the scope of this study) is, in an experiment that creates larger distances between adjacent nuclei, whether cells can create two distinctive lateral myosin clusters on the two sides of the same lateral membrane, or whether the formation of myosin clusters across the same membrane are coordinated?

3. The centrifugation experiment presented in Fig. 3 was a very elegant approach; however, more experimental details and control experiments should be provided to further validate the method. Firstly, it should be documented how centrifugation can affect the position of the nuclei in the absence of cell shape change and tissue movement. For example, how much basal nuclei movement can be achieved if the embryos are centrifuged during the cellular blastoderm stage? Secondly, when comparing the lateral myosin distribution between the centrifuged embryos and the control embryos, the authors should demonstrate whether the extent of apical constriction was comparable between the two conditions. This information is important given that the nuclei position is tightly correlated with the apical cell area. In addition, it was unclear whether a harsh treatment like centrifugation will change other properties, such as the number of cells undergoing apical constriction or the extent of cell lengthening during apical constriction. Finally, it was unclear why the impact of centrifugation on nuclei

positioning was spatially heterogeneous for the cells on the same side of the embryo (illustrated in Supplemental Fig. 5), as one would expect that the centrifugal force should be uniformly applied.

4. The authors use laser ablation to disrupt apical myosin in order to prevent cytoplasmic flow and in turn basal migration of the nucleus. They further used centrifugation to achieve the opposite effect, moving nuclei more basally. It would be beneficial for the authors to do the same centrifugation experiment and test the situation where the embryos are positioned with their ventral side facing the center of the rotor to keep the nuclei near the apical side during ventral furrow formation. This experiment would show how a lack or delay of nuclear migration affects the formation and positioning of lateral myosin clusters with an approach that does not disrupt the apical actomyosin network.

5. In Fig. 3g, the measurements indicate that lateral myosin clusters could be detected when the apex-to-nucleus distance was as small as around 10 microns, whereas in Fig. 2e, the data suggest that no lateral myosin clusters were formed in the cut region when the nuclei were apically positioned. Is there a threshold apex-to-nucleus distance that would allow the formation of lateral myosin clusters?

6. Regarding the role of microtubules (MTs) in the formation of lateral myosin clusters, the authors focused on the microtubules emanating from the centrosomes. However, the authors do not address the potential role of other MTs besides centrosomal MTs in lateral myosin regulation. A previous study demonstrated that there is a population of Patronin-mediated, minus-end stabilized noncentrosomal microtubules, where their minus ends are anchored at the apical surface (PMID: 31227595). Could these microtubules function to distribute RhoGEF2 to the lateral cortex? To what extent the effect of Colcemid on lateral myosin is due to the loss of noncentrosomal microtubules? Does Patronin play any role in the formation of lateral myosin clusters?

7. Certain aspects of the CytoSIM simulation appear to be counterintuitive. In the simulation presented in Fig 4g-i, the MT plus ends show higher probability density at the apical cytoplasmic – nucleus boundary in panel g (when the nucleus is more apical) than in panel h (when the nucleus is more basal). However, there is an apparent discrepancy between this result and the observation that EB1 showed lower cortical/cytoplasm ratio when nucleus is apically localized (Fig 4a, b).

8. The computer model presented in Fig 4 leads to a very interesting and surprising prediction that basally localized nucleus and centrosomes are sufficient to cause an increase in lateral EB1 intensity regardless of developmental stages (although this might be not true for RhoGEF2, since the lateral recruitment of RhoGEF2 also depends on T48). Could this prediction on EB1 be further tested by examining EB1 localization in mutant situations where the nuclei show higher variation in their apical-basal location (e.g., the Charleston mutant, PMID: 16421189)?

Minor points

1. The authors refer to the nuclei as an “impassable piston”, which should be softened because this statement is not necessarily definite. The word “piston” appears to indicate that there is no mixing between the cytoplasmic compartments separated by the nucleus. However, it has been previously shown that lipid droplets are able to transport past the nuclei from the basal compartment to the apical compartment near the onset of gastrulation, PMID: 14521831).

2. There appears to be some discrepancy in the localization and morphology of lateral myosin during gastrulation. For example, in Fig 1c, lateral myosin appears as elongated bars, each occupying a substantial apical-basal distance. In contrast, in Supplemental Fig 1d, lateral myosin appears as condensed puncta between the nuclei and the apical cortex. The difference should be explained. In addition, for the scenario demonstrated in Fig 1c, how was the apical-basal position of lateral myosin determined in the quantifications?

3. The explanation of the RhoGEF2 overexpression experiment (Supplementary Fig 2g) seems a little ambiguous – since RhoGEF2 activates the formation of lateral myosin cluster, it is unclear whether the enhanced formation of myosin cluster in embryos overexpressing RhoGEF2 is due to enhanced apical constriction, or due to the presence of more RhoGEF2 available to trigger lateral myosin activation.

4. The authors should clarify whether the optogenetic RhoGEF2 constructs recruit the full-length RhoGEF2 or just the functional domain of RhoGEF2. Based on the cited literature, it appears to be the latter.

5. In the simulation, is the extent to which centrosomal microtubules can pass through the space between the nucleus and the cell membrane dependent on the persistence length of the microtubules? It would be helpful to show how changing this parameter would alter the outcome.

6. Fig 1f: the meaning of individual data point in the main plot versus the inset should be more clearly defined.

7. Fig. 2: It appears that laser ablation was imposed after the formation of the apical actomyosin network in Fig. 2a and 2b but started before the onset of gastrulation in Fig. 2c, which causes confusion. The timing of the laser treatment should be more clearly indicated in the figure or the figure legend.

8. Fig 2e: the text says, “nuclei in the control zone move significantly more basally compared to nuclei in non-ablated embryos preserving or even enhancing the formation of MyoII-LCs (Line 131 – 132). However, Fig 2e shows that lateral myosin intensity is not significantly different between the control region and non-ablated embryos.

9. Fig 2f: the meaning of the x-axis label “apical cell surface ratio” is ambiguous. Based on the figure legend, it seems to indicate “change in apical cell surface”, which is a clearer description.

10. Fig 4f: the meaning of the numbers in the parentheses should be indicated.

11. Supplementary Fig 1b and c: the meaning of “N = 45 junctions” in the context of apical myosin intensity measurement is unclear.

12. Supplementary Fig 1c: it is unclear whether the plot represents a single measurement or the average of multiple measurements. If it is the latter, error bars should be added to the plot, and it would be helpful to show a few examples of individual measurements.

13. Supplementary Fig 2i: the stage of the embryo should be indicated in the figure legend.

14. Supplementary Fig 5b: The magenta lines should be defined in the figure legend.

15. Supplementary Fig 7a: it appears that some nuclei fused together at t = 3 – 9 min. Is this real or an imaging/visual artifact? In addition, the markers used to visualize nuclear envelopes should be indicated in the figure/legend.

16. Supplementary Fig 9c: the definition of time zero should be indicated.

17. Supplementary Fig. 9g: the stage of the embryos should be indicated.

18. Line 68, 110, 205, 293: it seems to be more appropriate to use “Overall” instead of “Finally”.

19. Line 105: 2016 should be 2014.

20. Line 260 – 263: the text stated that “More specifically, a great number of cells fail to apically constrict, phenocopying mesoderm cells from embryos injected with a 5mM ROCK-inhibitor Y-27632” However, the authors did not show whether 5 mM Y-27632 injection resulted in heterogeneous apical constriction as in the case of Colcemid injected embryos or merely caused a uniform delays/rate reduction in apical constriction.

21. Line 274: “rise” should be “raise”.

22. Supplementary Materials Line 40: “).” is missing at the end of the sentence.

23. Supplementary Materials Line 55: a space is missing in “fromlaser”.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Remarks to the Author:

The authors have made significant improvements to the manuscript by incorporating new quantifications, clarifications, and experimental data. I also understand the difficulty with some experiments and the concern with pleiotropic effects through long-term depletion. The alternative strategies provide convincing data to prove the author's point, and I fully support publication at this stage.

Reviewer #2

(Remarks to the Author)
The authors have thoroughly and meticulously addressed all of my previous comments. I now fully support the publication of this elegant piece of work in Nature Communications.

Nuclear position controls the activity of cortical actomyosin networks powering simultaneous morphogenetic events

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Université Côte d'Azur, CNRS, Inserm, iBV, Nice, France

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ANSWER TO REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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Overall, the authors have addressed most of the immediate questions experimentally, and the manuscript is well-written. Additionally, the methodology used in the paper is of a high standard. However, some results remain a bit hard to interpret at this stage, and additional controls would be needed.

We thank Reviewer#1 for her/his feedback. Here below our answers point-by-point.

Major Comments

1. Molecular Link Between Nuclear Movement and Microtubules

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We understand Reviewer#1 alternative hypothesis based on apical-to-basal junction relocation. Nevertheless, during apical constriction, Par3/6 dependent adherens junctions do not relocate more basally. On the contrary, they are further moved to the very apical side of mesoderm cells (Weng and Wieschaus JCB 2016). This *per se* rules out the apical-to-basal junction relocation hypothesis proposed by Reviewer#1.

A previous paper published by our team shows that the coalescence of the lateral actomyosin network on the lateral side of mesoderm cells (forming MyoII-LCs) induces clustering of E-cadherin and therefore the formation of another set of adherens junctions along the cell lateral sides (a 2nd

junctional tier - John and Rauzi DevCell 2021, Fig.3 and Fig.6I). In short, formation of the 2nd junctional tier is a consequence of cell lateral actomyosin contractions. With this in mind, we re-contextualized Reviewer#1 suggestion and monitored the effect of blocking nuclear migration on the 2nd junctional tier by quantifying E-cadherin. Our new analysis shows that nuclear migration impairment significantly reduces both MyoII and E-cad on the lateral side of cells, as expected. This new data is now included in **Supplementary Figure 7f and g** and presented in line 229.

2. RhoGEF2 Activation and EB1 Polarity

RhoGEF2 gets activated in very specific foci at the lateral site. Although more MT-plus ends interact with the lateral sides upon nuclear translocation, there is no polarity of EB1 movement before RhoGEF2 localization to the intercalating D-V junction. The authors show in Supp. Fig 10, e, f the similar localization of EB1, but this is observed only after the shrinkage of the intercalating D-V junction. Shouldn't EB1 density precede RhoGEF2 on this junction? It would be beneficial to image EB1 (GFP) together with RhoGEF2 or at least with MyoII (Cherry) to correlate the localization of both. The current results suggest additional molecular players regulate RhoGEF2. Also, it would be insightful to see the formation of lateral MyoII clusters using genetic perturbation of microtubules. For example, removing centrosomes by knocking down sas-4 or asl could provide a clearer picture of microtubule function compared to the complete removal of microtubules by colcemid.

We thank Reviewer#1 for her/his comments and suggestions. To dissect the precise temporal sequence of EB1 protein distribution along the lateral side of mesoderm cells, we now analyze EB1 intensity with higher temporal resolution. Our analysis shows that EB1 is preferentially enriched along DV junctions also prior to cell intercalation. This evidence is now presented in **Supplementary figure 12e and f**. In addition, we also tried to image both EB1 and RhoGEF2 simultaneously (as in de Las Bayonas et al. DevCell 2019). Unfortunately, we did not succeed as the *Drosophila* strain was extremely weak and kept dying (we got confirmation from Garcia de Las Bayonas that it is indeed a very unhealthy strain). Alternatively, we monitored tubulin and RhoGEF2 distribution simultaneously during intercalation at the lateral cortex. Our data show spatiotemporal correlation between the two proteins, confirming the hypothesis that microtubules participate to the delivery of RhoGEF2 to intercalating junctions. This new data is presented in **Supplementary Figure 12g**.

We agree with Reviewer#1 that genetic perturbation could complement our opto-drug and colcemid live injection based experiments to perturb microtubules. Nevertheless, genetic perturbation of microtubules in general lacks temporal specificity and therefore it can hinder with key processes occurring before mesoderm invagination (such as cellularization) that are necessary for ventral fold formation. For this reason, we favored temporally-resolved techniques (i.e., opto-disruption of microtubules and live-injection of Colcemid).

Regarding more specifically Sas-4 and Asterless mutations, unfortunately these are not viable at our stage of interest (Asl mutants lead to embryonic lethality - PMID: 17935995 - and DSas-4 mutants only display effects on centrosome organization after gastrulation has occurred - PMID: 16814722).

As an alternative technique we tested centrosome ablation by using infrared femtosecond laser ablation with sub-micrometer precision and sub-second time resolution (Rauzi and Lenne Methods Mol Biol. 2015). Nevertheless, this technique was unsuccessful because of rapid centrosome movement in 3 dimensions and fast repair, rendering the analysis of multiple ablation-induced centrosome disruption unfeasible.

To try our best to provide concreate feedback to Reviewer#1 suggestion on this point, we decided to undertake a complementary strategy. Previous work has shown that the acentrosomal pool of microtubules can be disrupted by genetically downregulating Patronin (Booth et al. DevCell 2014 and Ko et al. JCB 2019). We now used Patronin RNAi to unravel the role of the centrosomal versus the acentrosomal microtubule network in the formation of MyoII-LCs. Overall, Patronin downregulation did not abolish MyoII lateral recruitment (**Figure 11a and b**). This supports the idea that centrosomal and acentrosomal microtubules are sufficient and not necessary to form MyoII-LCs, respectively. This is now presented in line 310.

Here follows a more detailed description of the Patronin RNAi phenotype that we have now added to our study. Previous work has shown that Patronin RNAi partially affects apical constriction of

mesoderm cells (Ko et al. JCB 2019). Therefore, we quantified nuclear position and MyoII-LC formation in this mutated background. Our analysis shows that overall nuclei have a very heterogeneous apicobasal distribution resulting from groups of cells undergoing apical constriction and other groups of cells failing to apically constrict (**Figure 11c**). Remarkably, in the regions where nuclei migrate basally, MyoII-LCs are still able to form. We also quantified the respective position of both MyoII-LCs and nuclei. Our analysis shows that the "halfway rule" is satisfied also in the Patronin RNAi mutant embryos (**Figure 11d**). Based on these results, we propose that the acentrosomal microtubules are not necessary to drive the formation of MyoII-LCs. Nevertheless, this does not exclude the possibility that the acentrosomal microtubule network still contributes to MyoII-LC formation. A plausible hypothesis is that centrosomal microtubules are much more numerous than acentrosomal microtubules and therefore are sufficient to drive the formation of MyoII-LCs.

Conclusion

With these additions, I would be very supportive of the publication. The manuscript is convincing overall, but addressing the points above would strengthen the conclusions and provide a more comprehensive understanding of the mechanisms involved.

We thank again Reviewer#1 for her/his comments and feedback that has helped improving the paper.

Reviewer #2 (Remarks to the Author):

This well-written and elegant manuscript presents significant and timely research aimed at explaining how tissues undergo multiple concomitant shape changes during mesoderm folding and extension in the early Drosophila embryo morphogenesis. Previous studies demonstrate that this process requires specifically timed reorganization of cortical myosin in two separate tiers. The first tier of actomyosin localizes to the apical cell cortex and drives apical constriction of the cells, while the second tier localizes to the lateral cell cortex and drives cell intercalation for simultaneous tissue folding and convergence-extension. In this study, the authors present evidence that the nucleus acts as a barrier to shield the lateral cortex from the microtubule network so that the recruitment of RhoGEF2, a key activator of actomyosin, to the lateral cortex is regulated both spatially and temporally. By manipulation of the nucleus in gastrulating embryos, the authors demonstrate that the accumulation and position of lateral actomyosin is tightly associated with the apical-basal position of the nucleus. Furthermore, by combining pharmacological and optogenetic manipulations with computer modeling, the authors propose a model where the cytoplasmic flow caused by apical constriction passively relocates the nucleus and unshields the cells' lateral cortex for RhoGEF2 delivery by microtubules (MT), in order to designate the formation of the second actomyosin tier. Finally, the authors show evidence that this mechanism requires the coordinated function of the MT plus-end binding protein EB1 and the transmembrane protein T48, a developmentally regulated cortical anchor for RhoGEF2.

This study provides a new perspective on the nucleus as a spatiotemporal cytoskeleton compartmentalizer that drives part of the morphogenic process mediating coordinated epithelial folding and tissue extension. These results are important and worthy of publication as they offer a more holistic view of morphogenesis by deciphering how tissues undergo various simultaneous shape changes during morphogenesis and how cellular organelles can change position to provide spatial regulation of the force-generating machinery in the cell. The authors employed multiple approaches to test their main conclusions, including computer modeling, which is a significant strength of the paper. The data are well-quantified and thoroughly analyzed. However, for certain methods used in the study, additional control experiments are necessary to accurately assess the actual effects of the treatment. These controls

would strengthen the validity of the conclusions and provide a clearer understanding of the treatment's impact.

We thank again Reviewer#2 for her/his comments that have helped improving the paper and we would like to felicitate her/him for the outstanding revision work she/he has done. Here below our point-by-point answers to the comments.

Major points

1. In the experiments where the authors used laser ablation to dissect the apical actomyosin network (illustrated in Figure 2a and 2b), the apical area of the cells was greatly dilated after laser dissection, causing profound changes in cell shape. The authors should rule out the possibility that the reduction of lateral myosin intensity is due to dilution of the cortical signal as the cells were stretched, or due to a shift of myosin enrichment to a more apical location on the lateral cortex. In addition, to rule out a potential direct impact of laser ablation on lateral myosin, in particular in those experiments where laser ablation was iteratively applied to the embryos, the authors should show evidence that the lateral myosin is still present immediately after the laser treatment but disappears over time as the nuclei migrate apically. Finally, in the laser-dissected region, it appears that there were still some nuclei localized at a basal position comparable to some nuclei in the uncut control region (as illustrated by the partially overlapped distributions of apex-to-nucleus distance shown in Fig. 2d). Could lateral myosin be detected in the vicinity of these more basally localized nuclei in the dissected region?

We thank Reviewer#2 for her/his insightful comments that are very much appropriate. We now analyzed cell deformation in great detail. Firstly, our analysis shows that, while apical areas are greatly dilated upon ablation, changes in cell surface are significantly less pronounced at 10 µm from the apical surface (**Supplementary Figure 2g and h**). To directly test the "cortical dilution" hypothesis, we monitored junction length apically and laterally to see how laser ablation impacted cell cortices. We show that, due to the strong anisotropy of tension in the mesoderm tissue, tissue recoil is predominantly stretching AP junctions rather than DV junctions (the latter being where MyoII is laterally enriched). More importantly, DV junctions do not undergo more stretching than junctions in the control region (refer to the new **Supplementary Figure 2i**). This rules out the "cortical dilution" hypothesis. This is now presented in line 135.

To test if ablation leads to a shift of MyoII to more apical locations, we monitored MyoII signal along the entire apicobasal axis of mesoderm cells in both control/cut regions of ablated embryos and control embryos. Our analyses show that inhibition of nuclear displacement does not shift MyoII to other apicobasal locations (**Supplementary Figure 2c and c'**).

To directly test if laser ablation targeting the apical actomyosin network directly affects lateral MyoII, we now complement our initial control experiment (showing that ablation was restricted to the apical side and did not impact MyoII signal 10 µm from the apical surface - **Supplementary Figure 2d**) with a new experimental evidence and associated analysis. Monitoring MyoII signal before apical movement of nuclei upon DV ablation, as proposed by Reviewer#2, is technically non-feasible as cell shape changes and nuclear ectopic displacement occur in very short time scales (in the range of seconds). Therefore, we devised an alternative experimental solution that consist in laser ablating the apical network with minimal cell apical surface dilation in order to avoid nuclear migration back to the apical side and consequent MyoII-LC inhibition. To achieve this, we took advantage of the fact that tension in the mesoderm tissue is anisotropic with higher tension along AP and lower tension along DV (Martin et al. JCB 2010 and Fierling et al. Nature Comm 2022). We thus performed laser cuts along the AP axis (instead of DV) at an advanced stage of ventral furrowing. This results in apical network ablation with minimal cell surface dilation and nuclear apical relocation (Supplementary Figure 3e and f). In this context, we monitored MyoII along the lateral cortex and did not observe significant changes in MyoII density (**Supplementary Figure 4a, b, c and d**), validating once again the spatial precision of our ablation technique. More importantly, this new experiment provides direct evidence that the apical actomyosin network is *per se* not necessary to maintain MyoII-LCs.

Regarding the last point, we did not observe any recruitment of MyoII in these nuclei whose positioning overlapped with control nuclei (3 nuclei in total). It is important to notice that these nuclei

were flanked by apically located nuclei in the cut region. Therefore, given the small number of cases, we can speculate that MyoII lateral cluster formation is a non-cell-autonomous process and that RhoGEF2 inputs by both neighboring cells is required to form a MyoII-LC.

2. The evidence supporting the halfway rule (model 3) for lateral myosin localization should be strengthened further, or the conclusion should be softened. In Fig. 3b, the standard deviation for model 3 is not markedly different from model 2, where the position of lateral myosin is determined by the most basal nuclei of the four. Was the difference between the two models statistically significant? Additionally, the observations presented in the remaining parts of Fig. 3 (Fig. 3c – g) did not provide further distinction among the proposed models. It might be helpful to examine conditions where the neighboring nuclei have more distinct apical-basal locations to further test the halfway rule. For example, the author could examine the cells located at the border of the cut and uncut zones. Alternatively, the authors could examine nuclei in charleston (char) mutants, where there are irregularities in the alignment of the nuclei (Char is a regulator of nuclear morphogenesis and apical nuclear anchoring, and its RNAi embryo has wildtype microtubule organization and association to the nuclear envelope. PMID: 16421189). An interesting question (which might be beyond the scope of this study) is, in an experiment that creates larger distances between adjacent nuclei, whether cells can create two distinctive lateral myosin clusters on the two sides of the same lateral membrane, or whether the formation of myosin clusters across the same membrane are coordinated?

We thank Reviewer#2 for this comment that encouraged us to further test and analyze the data supporting the "half way" rule. We performed F-test between the standard deviations and effectively did not observe a statistical difference between the "average nucleus" and "most basal nucleus" conditions. To further distinguish the 3 models, we measured the R^2 in all three conditions. Our new analysis shows that the "average nuclear" condition delineates the best model having the highest R^2 (**Supplementary Figure 6f**). We agree with Reviewer#2 that analyzing conditions with nuclear variability would help to further support the "half way" rule. While we could not detect MyoII-LCs specifically located at the interface between the ablated and non-ablated region, we performed alternative analyses. We analyzed the inter-nucleus depth in the three experimental conditions in wild type genetic background previously used (i.e., control, delayed and centrifuged). Our new analysis shows that the difference in depth between neighboring AP nuclei can reach 15 µm in these conditions (**Supplementary Figure 6g**). This *per se* shows that the halfway rule is valid in control conditions, where the distribution of adjacent nuclei is inherently heterogeneous at the time of MyoII-LC recruitment. Furthermore, we identified pairs of AP nuclei displaying high variability in depth (defined as inter-nucleus depth > 5 µm, red-contoured data points in **Supplementary Figure 6g**) and could show that these nuclei still obey the halfway rule with most of the MyoII-LCs being established at half the distance of the two adjacent AP nuclei, despite the high variability in nuclear depth (**Supplementary Figure 6g, inset - Control**). This is now presented in line 197. As suggested by Reviewer#2 we also considered using Char-RNAi embryos. Nevertheless, during the first minutes of gastrulation in a Char-RNAi background, mesoderm nuclei show a position-variability not much different than in wild type embryos (WT-std = 0.2688 µm and Char-std = 0.3233 µm).

Finally, previous work from our lab identified a coordinated coalescence of actomyosin networks at the interface between two cells during the formation of a MyoII-LC (please view Fig. 2E, SFig. 1C and Video 2 right panel of the paper John and Rauzi DevCell, 2021). In addition, we now reflected on the fact that we never saw multiple MyoII-LCs on one same interface despise the heterogeneity in nuclear depth that can be observed also between adjacent cells in wild type conditions. This is now presented in line 193. Altogether, this indicates that the formation of MyoII-LCs is indeed coordinated and supports our model of the "halfway rule".

3. The centrifugation experiment presented in Fig. 3 was a very elegant approach; however, more experimental details and control experiments should be provided to further validate the method. Firstly, it should be documented how centrifugation can affect the position of the nuclei in the absence of cell shape change and tissue movement. For example, how much basal nuclei movement can be achieved if

the embryos are centrifuged during the cellular blastoderm stage? Secondly, when comparing the lateral myosin distribution between the centrifuged embryos and the control embryos, the authors should demonstrate whether the extent of apical constriction was comparable between the two conditions. This information is important given that the nuclei position is tightly correlated with the apical cell area. In addition, it was unclear whether a harsh treatment like centrifugation will change other properties, such as the number of cells undergoing apical constriction or the extent of cell lengthening during apical constriction. Finally, it was unclear why the impact of centrifugation on nuclei positioning was spatially heterogeneous for the cells on the same side of the embryo (illustrated in Supplemental Fig. 5), as one would expect that the centrifugal force should be uniformly applied.

We refined our methods to provide greater detail on the centrifugation protocol, including references to the employed techniques and the proposed mechanism. Nuclear positioning in control and centrifuged embryos at the end of cellularization was quantified and is presented in **Supplementary Figure 6c**. To assess the tissue-scale effects of centrifugation, we compared the number of rows of constricting cells in the mesoderm tissue between control and centrifuged embryos, finding no significant differences (**Supplementary Figure 6d**). We currently lack a definitive explanation for the heterogeneous phenotype observed following centrifugation. One possibility is that tissue curvature, combined with flow patterns generated during centrifugation, may result in non-uniform nuclear displacement. We also monitored the apical surfaces across conditions at the time of second-tier formation in control and centrifuged embryos and our analysis revealed that nuclear positioning and apical cell surface remain correlated, with enhanced nuclear displacement associated with a reduction in apical cell surface area, as anticipated (**Supplementary 6e**).

4. The authors use laser ablation to disrupt apical myosin in order to prevent cytoplasmic flow and in turn basal migration of the nucleus. They further used centrifugation to achieve the opposite effect, moving nuclei more basally. It would be beneficial for the authors to do the same centrifugation experiment and test the situation where the embryos are positioned with their ventral side facing the center of the rotor to keep the nuclei near the apical side during ventral furrow formation. This experiment would show how a lack or delay of nuclear migration affects the formation and positioning of lateral myosin clusters with an approach that does not disrupt the apical actomyosin network.

We actually had the same idea proposed by Reviewer#2. Nevertheless, we rapidly realized that this experiment is technically not feasible. Since the nuclei are already located apically, the idea would be to keep them apically during ventral fold formation by using the G force. The problem is that nuclear movement, MyoII-LC formation and time of centrifugation are all within the same time order. Therefore, the embryo would need to be imaged while being centrifuged. Unfortunately, this is technically not feasible for now with the tools presently available in our lab. As an alternative, we have performed basal-optogenetics to ectopically constrict cells at their basal side and impair nuclear movement without disrupting the apical actomyosin network (**Supplementary Figure 3**).

5. In Fig. 3g, the measurements indicate that lateral myosin clusters could be detected when the apex-to-nucleus distance was as small as around 10 microns, whereas in Fig. 2e, the data suggest that no lateral myosin clusters were formed in the cut region when the nuclei were apically positioned. Is there a threshold apex-to-nucleus distance that would allow the formation of lateral myosin clusters?

We thank Reviewer#2 for highlighting this interesting point. We do not observe MyoII-LCs between the apex of cells and 4 µm. We could speculate that any MyoII recruitment in the vicinity of the subapical /apical cortex will lead to their fusion apically, as the apical junctional pool of MyoII would then "suck in" the laterally recruited MyoII by advection (as described by the Weng and Wieschaus JCB 2016 paper, showing the formation of the apicolateral junctions). Based on our predictive model and *in vivo* observations, we thus estimate that a minimal apex-to-nucleus distance must be reached to allow MyoII-LC formation.

6. Regarding the role of microtubules (MTs) in the formation of lateral myosin clusters, the authors focused on the microtubules emanating from the centrosomes. However, the authors do not address the potential role of other MTs besides centrosomal MTs in lateral myosin regulation. A previous study demonstrated that there is a population of Patronin-mediated, minus-end stabilized noncentrosomal microtubules, where their minus ends are anchored at the apical surface (PMID: 31227595). Could these microtubules function to distribute RhoGEF2 to the lateral cortex? To what extent the effect of Colcemid on lateral myosin is due to the loss of noncentrosomal microtubules? Does Patronin play any role in the formation of lateral myosin clusters?

We thank Reviewer#2 for encouraging us to do these additional experiments to expand the paper. We now performed live imaging of cell lateral MyoII in Patronin RNAi embryos. Remarkably, Patronin downregulation *per se* does not abolish MyoII-LCs. More specifically, we observe defects in the apical MyoII distribution (in agreement with Ko et al. JCB 2019) with embryos showing a more or less severe phenotype (**Supplementary Figure 11a**). Overall, MyoII-LCs are preserved in parts of the mesoderm that undergo non-defective apical constriction and consequent nuclear displacement (**Supplementary Figure 11b and c**). We also quantified the respective position of MyoII-LCs and nuclei and observed that the halfway rule is still fulfilled (**Supplementary Figure 11d**). These evidences support the idea that the acentrosomal pool is unnecessary *per se* for MyoII-LC formation. Nevertheless, we cannot exclude the possibility that the Patronin dependent microtubules yet contribute to the formation of MyoII lateral clusters. If they do, to which extent they contribute remains to be addressed. A possible way to tackle this, is to count the number of centrosomal versus acentrosomal microtubules within a defined time period (for instance, by tracking EB1 comets moving towards the cell apical and basal side, respectively). To that end, live imaging with enhanced spatial and temporal resolution (going beyond what we can do at present) would need to be implemented along embryo sagittal or cross sections. If the number of centrosomal microtubules is magnitudes higher, this could provide an explanation to why perturbation of the acentrosomal microtubules network does not significantly impact MyoII-LC formation. This is now presented in line 310.

7. Certain aspects of the CytoSIM simulation appear to be counterintuitive. In the simulation presented in Fig 4g-i, the MT plus ends show higher probability density at the apical cytoplasmic – nucleus boundary in panel g (when the nucleus is more apical) than in panel h (when the nucleus is more basal). However, there is an apparent discrepancy between this result and the observation that EB1 showed lower cortical/cytoplasm ratio when nucleus is apically localized (Fig 4a, b).

We are sorry for the misunderstanding. In Figure 4b we are measuring the EB1 cortical levels specifically along the lateral portion of the cortex (5 to 15 µm) that is initially flanked by nuclei. We now clarify this by improving the drawing to better illustrate how quantification was performed. In the same way, the model shows an increase in microtubule-to-cortex contact density along the cell lateral size that is initially flanked by the nucleus (refer to **Figure 4g and h**).

8. The computer model presented in Fig 4 leads to a very interesting and surprising prediction that basally localized nucleus and centrosomes are sufficient to cause an increase in lateral EB1 intensity regardless of developmental stages (although this might be not true for RhoGEF2, since the lateral recruitment of RhoGEF2 also depends on T48). Could this prediction on EB1 be further tested by examining EB1 localization in mutant situations where the nuclei show higher variation in their apical-basal location (e.g., the Charleston mutant, PMID: 16421189)?

We do not agree with Reviewer#2 prediction that probably originates from an over interpretation of the computational molecular model. If one accepts the seemingly tautological idea that "to have an enrichment of MT plus ends at the cortex, it is necessary that MT plus ends reach the cortex", the model highlights the fact that, given the geometrical constraint imposed by the cell boundaries, nuclear displacement is necessary for MT plus ends to reach portions of the lateral cortex that would be otherwise physically inaccessible. Therefore, the model does not propose that nuclear displacement is

sufficient as it does not take into account the contribution of other possible molecular players that could, for instance, stabilize or severe MT plus end at the cortex or in other regions of the cell.

Minor points

1. The authors refer to the nuclei as an "impassable piston", which should be softened because this statement is not necessarily definite. The word "piston" appears to indicate that there is no mixing between the cytoplasmic compartments separated by the nucleus. However, it has been previously shown that lipid droplets are able to transport past the nuclei from the basal compartment to the apical compartment near the onset of gastrulation, PMID: 14521831).

There is a misunderstanding: here we are not referring to nuclei "as impassable pistons". We are referencing to the Gelbart et al. PNAS 2012 paper that proposes a model that assumes nuclei working like "impassable pistons" (see page 19302, right column and line 12th where this exact expression is used). We agree with Reviewer#2 that the "impassable" part of the assumption is inappropriate. We now highlight this point in the discussion (refer to line 382).

2. There appears to be some discrepancy in the localization and morphology of lateral myosin during gastrulation. For example, in Fig 1c, lateral myosin appears as elongated bars, each occupying a substantial apical-basal distance. In contrast, in Supplemental Fig 1d, lateral myosin appears as condensed puncta between the nuclei and the apical cortex. The difference should be explained. In addition, for the scenario demonstrated in Fig 1c, how was the apical-basal position of lateral myosin determined in the quantifications?

Figure 1c is only a representative image not used for quantification. Figure 1c was obtained by (i) performing a ventral tissue front-view z-stack (i.e., z-stack along the DV axis), (ii) rotating the stack to obtain a cross-section view and (iii) by performing a plane-projection along the AP axis. Therefore, MyoII-LCs appear more as "elongated bars" because of image processing in step (ii) resulting in a Y axis (corresponding to the z resolution of the initial z-stack) spatially less resolved. The same elongated artefact is shown in Gracia et al. NatureComm 2019. In addition, MyoII-LCs appear overlapping and at different apical-basal positions because of image processing in step (iii) (it should be noticed that the ventral region is curved also along AP). We used coalesced MyoII-LCs as read-outs from front-view z-stacks to perform the analysis/quantification. We now clarify this in the legend and in the method section.

3. The explanation of the RhoGEF2 overexpression experiment (Supplementary Fig 2g) seems a little ambiguous – since RhoGEF2 activates the formation of lateral myosin cluster, it is unclear whether the enhanced formation of myosin cluster in embryos overexpressing RhoGEF2 is due to enhanced apical constriction, or due to the presence of more RhoGEF2 available to trigger lateral myosin activation.

We now measured apical constriction in RhoGEF2 overexpressing embryos and show no statistical difference with control embryos (attached).

4. The authors should clarify whether the optogenetic RhoGEF2 constructs recruit the full-length RhoGEF2 or just the functional domain of RhoGEF2. Based on the cited literature, it appears to be the latter.

Reviewer#2 is perfectly correct. We now amend this in the corresponding method section "Optogenetic recruitment of RhoGEF2".

5. In the simulation, is the extent to which centrosomal microtubules can pass through the space between the nucleus and the cell membrane dependent on the persistence length of the microtubules? It would be helpful to show how changing this parameter would alter the outcome.

We thank Reviewer#2 for suggesting to test this other microtubule property in order to further extend our analyses. Unfortunately, persistence length cannot be modulated *per se*. Alternatively, we varied rigidity (as persistence length is proportional to rigidity/temperature). Remarkably, our new *in silico* analysis shows that rigidity plays an important role in controlling the distribution of MTs in our model. The new results are now shown in **Supplementary Figure 9l** and presented in line 287

6. Fig 1f: the meaning of individual data point in the main plot versus the inset should be more clearly defined.

The legend was modified for greater clarity.

7. Fig. 2: It appears that laser ablation was imposed after the formation of the apical actomyosin network in Fig. 2a and 2b but started before the onset of gastrulation in Fig. 2c, which causes confusion. The timing of the laser treatment should be more clearly indicated in the figure or the figure legend.

Ablation was performed after the onset of gastrulation. Crosses simply specify the position of the cuts and serve as a control to show that embryos do not exhibit differences in nuclear positioning in these regions, prior to the cut. We now clarified this in the legend.

8. Fig 2e: the text says, "nuclei in the control zone move significantly more basally compared to nuclei in non-ablated embryos preserving or even enhancing the formation of MyoII-LCs (Line 131 – 132). However, Fig 2e shows that lateral myosin intensity is not significantly different between the control region and non-ablated embryos.

Indeed, we do not observe differences in signal, mostly number of clusters, apico-basal position (Figure 3g) and distribution (as in-plane cable-like structures - Supplementary Figure 2f).

9. Fig 2f: the meaning of the x-axis label "apical cell surface ratio" is ambiguous. Based on the figure legend, it seems to indicate "change in apical cell surface", which is a clearer description.

We now changed this as suggested by Reviewer#2.

10. Fig 4f: the meaning of the numbers in the parentheses should be indicated.

We now added this to the legend.

11. Supplementary Fig 1b and c: the meaning of "N = 45 junctions" in the context of apical myosin intensity measurement is unclear.

We now corrected the legend.

12. Supplementary Fig 1c: it is unclear whether the plot represents a single measurement or the average of multiple measurements. If it is the latter, error bars should be added to the plot, and it would be helpful to show a few examples of individual measurements.

The average signal is acquired using 3 different embryos. We then compute one rate from that average signal to draw the curves. Computing different rates/embryo then showing the info+error bar doesn't change interpretation nor results but gives a much less smooth/visually understandable plot. That's why we opted to show the rate from average signal rather than the average rate from different embryos. This is now specified in the figure legend and methods.

13. Supplementary Fig 2i: the stage of the embryo should be indicated in the figure legend.

The stage was Stage 6 late cellularization. We now added it to the legend.

14. Supplementary Fig 5b: The magenta lines should be defined in the figure legend.

We now added details in the legend.

15. Supplementary Fig 7a: it appears that some nuclei fused together at t = 3 – 9 min. Is this real or an imaging/visual artifact? In addition, the markers used to visualize nuclear envelopes should be indicated in the figure/legend.

We used UbiGFP-nls to track nuclear signal and processed nuclear contours. The image is a Z-projection that creates visual artefacts. Any of those artefacts was excluded from analysis in single-stack analysis. We now clarify this in the method section.

16. Supplementary Fig 9c: the definition of time zero should be indicated.

T0 corresponds to the time of injection. We now added it to the legend.

17. Supplementary Fig. 9g: the stage of the embryos should be indicated.

Stage is gastrulation, during lateral MyoII recruitment. We now added to the legend.

18. Line 68, 110, 205, 293: it seems to be more appropriate to use "Overall" instead of "Finally".

We now amended the text as suggested.

19. Line 105: 2016 should be 2014.

We now amended the text as indicated.

20. Line 260 – 263: the text stated that "More specifically, a great number of cells fail to apically constrict, phenocopying mesoderm cells from embryos injected with a 5mM ROCK-inhibitor Y-27632" However, the authors did not show whether 5 mM Y-27632 injection resulted in heterogeneous apical constriction as in the case of Colcemid injected embryos or merely caused a uniform delays/rate reduction in apical constriction.

We now specify this and provide an example in the new **Movie 10**.

21. Line 274: "rise" should be "raise".

We thank Reviewer#2 for highlighting this spelling error that we now corrected.

22. Supplementary Materials Line 40: ")." is missing at the end of the sentence.

We thank Reviwer#2 for highlighting this typing error that we now corrected.

23. Supplementary Materials Line 55: a space is missing in "fromlaser".

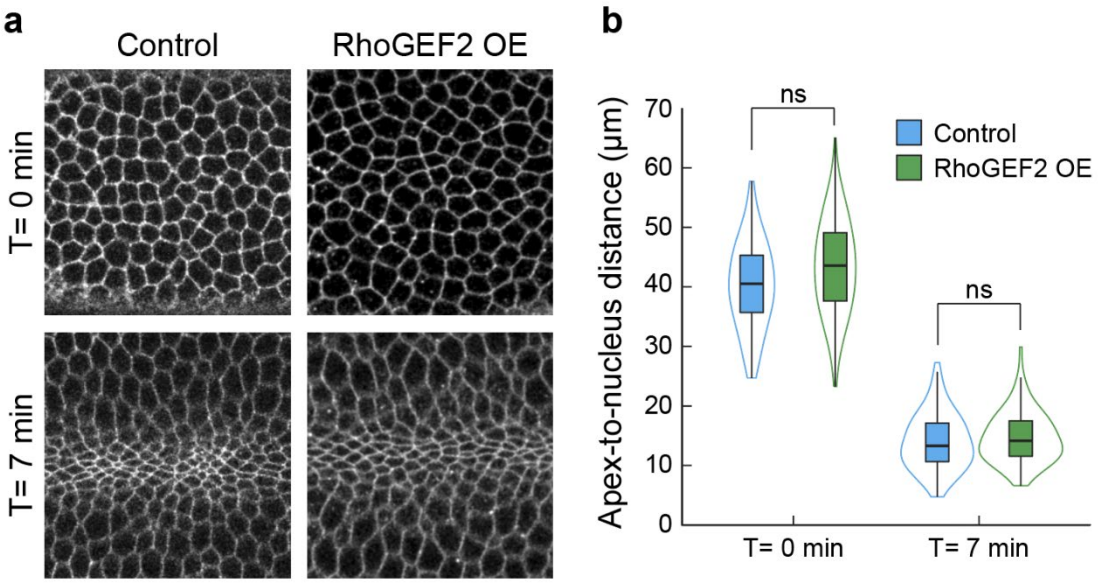
We thank Reviwer#2 for highlighting this typing error that we now corrected.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank Reviewer#3 for her/his revision work.

Attachments



Apical cell constriction in control and RhoGEF2 OE embryos. a, Front view of the apical side of mesoderm cells at the onset (top) and 7 min into gastrulation (bottom) in WT and RhoGEF2-overexpressing embryos. **b,** Quantification of apical cell surfaces in control and RhoGEF2-overexpressig embryos (n=6 embryos, N=781 cells).