Combinatorial patterns of graded RhoA activation and uniform F-actin depletion promote tissue curvature

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Reviewer 1

Evidence, reproducibility and clarity

In this manuscript entitled 'Combinatorial patterns of graded RhoA activation and uniform F-actin depletion promote tissue curvature' by Denk-Lobnig et al. the authors study the organisation of junctional F-actin during the process of mesoderm invagination during gastrulation in the model Drosophila. Following on from previous work by the same lab that identified and analysed a multicellular myosin II gradient across the mesoderm important for apical constriction and tissue bending, the authors now turn their attention to actin. Using imaging of live and fixed samples, they identify a patterning of F-actin intensity/density at apical junctions that they show is dynamically changing going into mesoderm invagination and is set up by the upstream transcription factors driving this process, Twist and Snail. They go on to show, using genetic perturbations, that both actin and the previously described myosin gradient are downstream of regulation and activation by RhoA, that in turn is controlled by a balance of RhoGEF2 activation and RhoGAP C-GAP inactivation. The authors conclude that the intricate expression patterns of all involved players, that all slightly vary from one another, is what drives the wild-type distinctive cell shape changes in particular rows of cells of the presumptive mesoderm and surrounding epidermis.

This is a very interesting study analysing complex and large-scale cell and tissue shape changes in the early embryo. Much has been learned over the last decade and more about many of the molecular players and their particular behaviours that drive the process, but how all upstream regulators work together to achieve a coordinated tissue-scale behaviours is still not very well understood, and this study add important insights into this.

The experiments seem well executed and support the conclusion drawn, but I have a few comments and questions that I feel the authors should address to strengthen their argument.

General points:

1. The authors state early on that they chose to focus on junctional rather than apical medial F-actin, but it is unclear to me really what the rationale behind that is. In much of the authors earlier work, they study the very dynamic behaviour of the apical-medial actomyosin that drives the apical cell area reduction in mesodermal cells required for folding. They have previously analysed F-actin in the constricting cells, but have only focused on the most constricting central cell rows (Coravos, J. S., & Martin, A. C. (2016). Developmental Cell, 1-14). The role of junctional F-actin compared to the apical-medial network on which the myosin works to drive constriction is much less clear, it could stabilize overall cell shape or modulate physical malleability or compliance of cells, or it could more actively be involved in implementing the 'ratchet' that needs to engage to stabilise a

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shrunken apical surface. I would appreciate more explanation or guidance on why the authors chose not to investigate apical-medial F-actin across the whole mesoderm and adjacent ectoderm, but rather focused in junctional F-actin, especially explaining better throughout what they think the role of the junctional F-actin they measure is.
2. Comparing the F-actin labeling in the above previous paper to the stainings/live images shown here, they look quite different. This is most likely due to the authors here not showing the whole apical area but focusing on junctional, i.e. below the most apical region. It is not completely clear to me from the paper at what level along the apical-basal axis the authors are analysing the junctional F-actin. Supplemental Figure 2 seems to suggest about half-way down the cell, which would be below junctional levels. Could the authors indicate this more clearly, please? Overall, I would appreciate if the authors could supply some more high- resolution images of F-actin from fixed samples (which I assume will give the better resolution) of how F-actin actually looks in the different cells with differing levels. Is there for instance a visible change to F-actin organisation? And could this help explain the observed changes in intensity and their function?
3. Along the same lines of thought as in point 2): Dehapiot et al. (Dehapiot, B., ... & Lecuit, T. (2020). Assembly of a persistent apical actin network by the formin Frl/Fmnl tunes epithelial cell deformability. Nature Cell Biology, 1-21) have recently shown for the process of germband extension and amnioserosa contraction that two pools of F-actin can be observed, a persistent pool not dependent on Rho[GTP] and a Rho-[GTP] dependent one. Could the authors comment on what they think might occur in the mesoderm, are similar pools present here as well?
4. As the authoirs state themselves, Rho does not only affect actin via diaphanous, but of course also myosin via Rock. So it would be good to refelect this more in the interpretation and discussion of data, as the causal timeline could be complex.

More specific comments to experiments and figures:

5. Reduction of junction function by alpha-catenin-RNAi: how strong is the reduction in catenin? Could they label a-catenin in fixed embryos? The authors conclude the original pre-constriction patterning of F-actin intensity is not dependent on intact junctions, but they show that the increase in F-actin in the mesodermal cells concomitant with apical constriction is in fact impaired in the RNAi. Thus, the authors can also not conclude whether the continued accumulation of myosin and its persistence depend on intact junctions. The initial set-up of the myosin gradient in terms of intensity distribution is unaffected, but clearly dynamics, subcellular pattern, interconnectivity etc. of myosin are affected and thus may well depend on some mechanical feed-back. I find this section of the manuscript slightly overstated and feel the conclusion should be more cautious.
6. Figure 1 versus Figure 2: Why do the Utrophin-ABD virtual cross-sections look so fuzzy and bad in comparison to phalloidin labelled F-actin in the virtual cross-section in Fig. 1B and C? The labelling shown in 2B and D does not even look very junctional...
7. Figure 5 C and D: the control gradients for myosin shown in C and D are completely different, for C the half-way height cell row is deduced as 5 whereas the (in theory identical) control measure in D has row 3 at halfway height! Why is this? Putting all curves together in the same panel would suggest that that C control curve is very similar to RhoGEF2-OE! This can't be right.
8. Still in Figure 5: Panels C and D again, but for apical area: are the control and C-GAP- RNAi or RhoGEF2-OE curves significantly different? What statistics were used on this?
9. Supplemental Figure 1: Panels in D: I appreciate this control, but would really also like to see the same control at a stage when folding has commenced and stretched cells are present at the margin of the mesoderm. How homogenous does the GAP43 label look in those?
10. Supplemental Figure 5: Panel 5 B: the authors conclude that the myosin gradient under RhoGEF2 RNAi is not smaller, but looking at the curves it in fact looks wilder. They also mention that the overall level of myosin in this condition is lower than the control...
11. Following on from the above, a comment of Figure 7: - The authors use RhoGEF2 RNAi stating that it affects the actin pattern, but the myosin pattern also seems affected. In line 318 the authors state that they use this condition to look at how junctional actin density affects curvature. I find this phrase misleading as It might lead the readers to think that RHoGEF2 RNAi only affects junctional F-actin, although it also affects myosin patterning.
- Line 311: confusingly, the authors state that an increase in the actomyosin gradient affects curvature.But it is only the myosin gradient that is increased, while the junctional actin gradient is

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flatter than the control in both C-GAP RNAi and RhoGEF2 OE (the distinction is even made by authors line 243). Could this be clarified?

Significance

Morphogenesis of organs, and how these highly coordinated processes are driven by transcriptional events, local control (of for instance cytoskeletal behaviour), is a major field in developmental and cell biology. Advances over the last decade have led to a much better understanding of the role of myosin (in the form of actomyosin) in defining cell and therefore tissue shape in morphogenesis. The role and control fo actin organisation, that the myosin depends upon for its action, is much less understood. Thus this study will add an important piece of understanding of the basic control of morphogenesis.

Reviewer 2

Evidence, reproducibility and clarity

Mesoderm invagination during Drosophila gastrulation has been a paradigm for how regionally restricted gene expression locally activates Rho signalling and for how subsequently activated acto-myosin drives cell shape changes which in turn lead to a change in tissue morphology. Despite the numerous studies on this subject and a good understanding of the overall process, several important aspects have remained elusive so far. Among these is the dynamics of cortical and junctional F-actin and its contribution to the shape changes of cells and tissue. Previous studies have focused on MyoII, the „active" component of the actin cytoskeleton. The dynamics of the „passive" counterpart, namely actin filaments, has been neglected, although it is clear that Rho signalling controls both branches.

1. Although I clearly acknowledge the efforts taken by the authors to define a function of cortical (junctional) F-actin in apical constriction and furrow formation, several central aspects of the study are not sufficiently resolved and conclusive. Rho signalling controls MyoII via Rok and F-actin via forming/dia, among other less defined targets. The role of MyoII and cortical contraction could be conclusively sorted out, since inhibition of Rok affects the MyoII branch but not the other branches. A similar approach, i. e. a specific inhibition/depletion without affecting the other branch, has not been taken yet for the F-actin branch. The authors have not resolved this issue. When analysing the mutants, the authors cannot distinguish the effect of Rho signalling on the MyoII and F-actin branch. For this reason the changes in F-actin distribution in the mutants are linked to changes in Myo activity and thus a function cannot be assigned to F-actin. In order to derive a specific role of F-actin distribution for furrow formation, the authors need to find experimental ways to affect F-actin levels without affecting MyoII, for example by analysing mutants for dia or other formins.
2. The authors employ a discontinuous spatial axis by the cell number. Although there are good arguments to understand and treat the cells as units, there are also good arguments for using a scale with absolute distance. I have doubts that the graded distributions presented by the authors are a result of this scaling with cell units. When looking at panel B of Fig 1 or Fig. 2A,B, for example, a sharp step like distribution is visible at the boundary between mesoderm and ectoderm anlage. In contrast a F-actin intensity distribution is graded after quantification. The graded distribution appears not to be a consequence of averaging because an even sharper step is very obvious in a projection along the embryonic axis as shown in panel B and D of Fig. 2, for example. The difference of a sharp step in the images and graded distribution after quantification with a spatial axis in cell number, is obvious for a-catenin in Fig. 3D and Rho signalling in Fig. 4 B. As the authors base their central conclusion (see headline) on the graded distribution, resolving the issue of spatial scale is a prerequisite of publication.
3. The authors put the spatial distribution of Rho signalling and F-actin into the center of their conclusion. They do so by affecting the pattern with mutants in twist/snail and varying upstream factors of Rho signalling. With respect to myo activation this have been done previously although possibly with less detail and it is no new insight that the width of the mesoderm anlage and corresponding Rho signalling domain has a consequence on the shape of the groove and furrow. To maintain the conclusion of the manuscript that spatially graded Rho signalling is contributes to tissue curvature, more convincing ways to change the pattern of Rho signalling are needed.

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Changing the balance of GEF and GAP shows the importance of Rho signalling and possibly signalling levels but not the contribution of its spatial distribution.

Significance

The question of a contribution of F-actin is addressed in this manuscript. The authors quantify F-actin in fixed and living embryos at two prominent steps in ventral furrow formation, (1) shortly prior to onset of apical constrictions and (2) when the groove has formed. They distinguish junctional and „medial" cortical F-actin. They employ a discontinuous spatial axis, the number of cells away from the ventral midline but not an absolute scale (see my notes below). The measurements are applied to wild type and mutant embryos affecting the transcriptional patterning (twist, snail), adherens junctions, and Rho signalling. The authors claim to reveal by their measurements a graded distribution of F- actin intensities with a peak at the ventral midline and a second peak at the boundary between mesoderm and ectoderm with a low point in the stretching cells of the mesectoderm. The authors further claim to reveal a graded distribution of Rho signalling components within the mesoderm anlage. Based on these data the authors conclude that graded Rho signalling and depletion of F-actin promote tissue curvature.

Reviewer 3

Evidence, reproducibility and clarity

Previous work has shown that mesoderm invagination at the ventral midline of the Drosophila embryo requires precise spatial regulation of actomyosin levels in order to fold the tissue. In this work, Denk-Lobnig and colleagues further investigate the spatial distribution of myosin and F-actin in the mesoderm and how these patterns are established.
The authors identify an F-actin pattern at the apical cell junctions that emerges upon folding, with elevated levels in the cells around the ventral midline, a decrease in junctional F-actin in the marginal mesoderm, and then an increase at the mesoderm-ectoderm border. They identify Snail and Twist as regulating different aspects of establishing this F-actin pattern. Additionally, by modulating RhoA activity (downstream of Twist) the authors are able to alter the width of the actomyosin pattern without affecting the width of the mesoderm tissue, which in turn affects the curvature of the tissue fold and the post-fold lumen size.

The authors have conducted an elegant quantitative analysis of the distribution of actin, myosin and several of their regulators across the tissue. The study makes an attempt at integrating a large amount of information into a model of tissue folding, and the concept of mechanical gradients is exciting and still underexplored. I am concerned that the interpretation of some results focuses on specific details but ignores larger scale effects (e.g. potential effects of some of the manipulations on the ectoderm, and the impact that that could have on tissue folding are largely ignored). The statistical analysis of several results should also be improved. I suggest to address the following points.

MAJOR

1. Line 127 and Figure 1E: The authors argue that there is an anticorrelation between F-actin distribution and cell areas. However, an R-squared value of 0.1083 rather suggests little-to-no correlation. The authors should evaluate the statistical significance of that correlation.

2. Figure 5: claims that the width of the actomyosin gradient is affected by the various perturbations should be supported with statistical analysis. For example, the half-maximal gradient position for each individual myosin trace could be calculated (instead of using the mean trace), displayed using a box plot, and tested for significance using the Mann-Whitney U test, as in Figure 7. This is slightly complicated by the fact that the control group in Figure 5C is the same as the control group in Figure 3E, which needs to be carefully considered. Also, similar calculations should be made for the F-actin data in Fig 5E-G since throughout the rest of the paper, the authors refer to the width of the "actomyosin gradient" which implicates both myosin and actin.

3. Line 142 and Figure 2B-C: I was confused by the description of the snail phenotype:

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- a. the claim that in snail mutants actin levels are uniform: based on Figure 2C, I'd say that F-actin levels decrease across the mesoderm moving away from the ventral midline, and that the main issue is with the accumulation of actin in the distal end of the mesoderm. The authors should better justify the claim that F-actin levels are uniform in snail mutants (or remove it). Maybe comparing F-actin levels in the first four or five rows of the mesoderm?
- b. how about the increase of F-actin in the distal mesoderm, just adjacent to the ectoderm boundary? Why is it gone in snail mutants?

4. With alpha-catenin-RNAi, F-actin depletion across the mesoderm still occurs, but junctional F-actin levels are not increased around the midline. While some explanations are offered in the text, the reason for this phenotype seems important for the story. The text in lines 204-205 suggests that F-actin that would normally be localized to the apical junctions is instead being drawn into medioapical actomyosin foci. Is this idea supported by evidence that medioapical F-actin in control embryos is lower than in alpha-catenin RNAi?
5. Figure 6A: there is a correlation between cell position and the productivity of myosin pulses, which the authors attribute to the RhoA gradient. This should be more definitively demonstrated by:
- a. Plot and calculate the correlation between RhoA levels (measured with the RhoA probe) and the change in cell area caused by a contraction pulse. Is this a significant correlation?
- b. How does myosin persistence change when RhoA is manipulated, e.g. in RhoA overexpressing embryos or in RhoA RNAi?

MINOR

1. The authors should indicate if the myosin shown in Figure 1A is junctional or medioapical. If it is junctional, does medioapical myosin better match junctional F-actin and cell areas? Similarly, if they are showing medioapical myosin, how does junctional myosin compare to junctional actin? It seems to me that consistently comparing the patterns of junctional F-actin and medioapical myosin (and RhoGEF2, RhoA, and ROCK in Figure 4) could be somewhat misleading, as the pools compared localize in different subcellular compartments.
2. Most of the intensity traces for myosin and F-actin are presented as normalized intensity, relative to the highest intensity in the trace. However, there are claims throughout the text about the relative levels of myosin (ex. Line 241) and F-actin (conclusions based on Fig. 2B-D) that should be supported by quantification. It seems that changes in intensity for both F- actin and myosin, in addition to shape of the gradient, would contribute to the understanding of actomyosin regulation in this tissue. However, if intensities cannot be directly compared between groups due to variation in imaging settings or staining protocols, there should be no claims made about changes in overall F-actin or myosin intensity.
3. The significance of the correlation in Figure 7E should be quantified.
4. Supplemental Figure 2: does the segmentation image match the second Z reslice immediately above? It does not appear so, or perhaps they are just not aligned. Having the two match would be more convincing of the segmentation technique.

Significance

The authors have conducted an elegant quantitative analysis of the distribution of actin, myosin and several of their regulators across the tissue. The study makes an attempt at integrating a large amount of information into a model of tissue folding, and the concept of mechanical gradients is exciting and still underexplored. I am concerned that the interpretation of some results focuses on specific details but ignores larger scale effects (e.g. potential effects of some of the manipulations on the ectoderm, and the impact that that could have on tissue folding are largely ignored). The statistical analysis of several results should also be improved.

First decision letter

MS ID#: DEVELOP/2020/199232

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MS TITLE: Combinatorial patterns of graded RhoA activation and uniform F-actin depletion promote tissue curvature
AUTHORS: Marlis Denk-Lobnig, Natalie C Heer, and Adam Martin

I apologise for not responding to you earlier. I have received and looked in detail at the reviewers comments and your response to these comments.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed according to your proposed plan.
I will look at the revision myself and the manuscript will not be sent back to reviewers of Review Commons.

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.

Author response to reviewers' comments

We thank the reviewers for carefully reading our manuscript. We found their comments to be incredibly thoughtful and constructive and greatly appreciate their feedback. We are confident that addressing the reviewers' concerns has strengthened our manuscript.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript entitled 'Combinatorial patterns of graded RhoA activation and uniform F- actin depletion promote tissue curvature' by Denk-Lobnig et al. the authors study the organisation of junctional F-actin during the process of mesoderm invagination during gastrulation in the model Drosophila. Following on from previous work by the same lab that identified and analysed a multicellular myosin II gradient across the mesoderm important for apical constriction and tissue bending, the authors now turn their attention to actin. Using imaging of live and fixed samples, they identify a patterning of F-actin intensity/density at apical junctions that they show is dynamically changing going into mesoderm invagination and is set up by the upstream transcription factors driving this process, Twist and Snail. They go on to show, using genetic perturbations, that both actin and the previously described myosin gradient are downstream of regulation and activation by RhoA, that in turn is controlled by a balance of RhoGEF2 activation and RhoGAP C-GAP inactivation. The authors conclude that the intricate expression patterns of all involved players, that all slightly vary from one another, is what drives the wild-type distinctive cell shape changes in particular rows of cells of the presumptive mesoderm and surrounding epidermis.

This is a very interesting study analysing complex and large-scale cell and tissue shape changes in the early embryo. Much has been learned over the last decade and more about many of the molecular players and their particular behaviours that drive the process, but how all upstream regulators work together to achieve a coordinated tissue-scale behaviours is still not very well understood, and this study add important insights into this.

The experiments seem well executed and support the conclusion drawn, but I have a few comments and questions that I feel the authors should address to strengthen their argument.

We thank the reviewer for their interest in the paper and their helpful comments.

General points:

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1. The authors state early on that they chose to focus on junctional rather than apical medial F-actin, but it is unclear to me really what the rationale behind that is. In much of the authors earlier work, they study the very dynamic behaviour of the apical-medial actomyosin that drives the apical cell area reduction in mesodermal cells required for folding. They have previously analysed F-actin in the constricting cells, but have only focused on the most constricting central cell rows (Coravos, J. S., & Martin, A. C. (2016). Developmental Cell, 1-14). The role of junctional F-actin compared to the apical-medial network on which the myosin works to drive constriction is much less clear, it could stabilize overall cell shape or modulate physical malleability or compliance of cells, or it could more actively be involved in implementing the 'ratchet' that needs to engage to stabilise a shrunken apical surface.
I would appreciate more explanation or guidance on why the authors chose not to investigate apical-medial F-actin across the whole mesoderm and adjacent ectoderm, but rather focused in junctional F-actin, especially explaining better throughout what they think the role of the junctional F-actin they measure is.

We focused on the junctional/lateral F-actin pool because this is where tissue-wide patterns in intensity variation across the mesoderm-ectoderm boundary are observed. Indeed, when we compare the apical-medial F-actin of marginal mesoderm cells to ectoderm cells in cross sections, we find no apparent difference, whereas there is a striking difference in junctional/lateral F-actin density (Fig. 1B, C; Supplemental Fig. 1A, D). We agree with the reviewer that this required added justification. Therefore, we have:
1) Provided higher resolution images of apical-medial F-actin comparing different regions of mesoderm and ectoderm (Figure S2A, B), 2) revised the text in lines 95-98, 104-111 to better justify why we chose junctional/lateral F-actin to focus our tissue-level analysis and to elaborate more on what we think the role of junctional/lateral F-actin in this process may be, and 3) added possible roles for lateral actomyosin to the Discussion, including a possible role in the ratchet mechanism.

2. Comparing the F-actin labeling in the above previous paper to the stainings/live images shown here, they look quite different. This is most likely due to the authors here not showing the whole apical area but focusing on junctional, i.e. below the most apical region. It is not completely clear to me from the paper at what level along the apical-basal axis the authors are analysing the junctional F-actin. Supplemental Figure 2 seems to suggest about half-way down the cell, which would be below junctional levels. Could the authors indicate this more clearly, please? Overall, I would appreciate if the authors could supply some more high-resolution images of F-actin from fixed samples (which I assume will give the better resolution) of how F- actin actually looks in the different cells with differing levels. Is there for instance a visible change to F-actin organisation? And could this help explain the observed changes in intensity and their function?

We apologize for the confusion, we were referring to 'junctions' as the lateral contacts between cells, as opposed to the adherens junctions at the apical surface. We have modified the text to use the term 'lateral' rather than 'junctional' F-actin, so as to avoid this confusion. The difference in cortical F-actin staining is not restricted to a particular apical-basal position, but extends along the length of the lateral domain (Fig. 1B, C). As far as we can tell the actin underlies the entire cell circumference and lateral surfaces.
We have: 1) revised the text in lines 573-587, 722 and 740 to better define the apical-basal position within the cell that we are showing, and 2) included high-resolution *en face* images of F-actin at apical and lateral/subapical positions, across different cell positions, in live and fixed embryos in Fig. S2A,B to better justify our focus on lateral F-actin.
Our data show a clear difference in lateral F-actin density across the mesoderm- ectoderm boundary, but no clear differences in F-actin structure.

3. Along the same lines of thought as in point 2): Dehapiot et al. (Dehapiot, B., ... & Lecuit, T. (2020). Assembly of a persistent apical actin network by the formin Frl/Fmnl tunes epithelial cell deformability. Nature Cell Biology, 1-21) have recently shown for the process of germband extension and amnioserosa contraction that two pools of F-actin can be observed, a persistent pool not dependent on Rho[GTP] and a Rho-[GTP] dependent one. Could the authors comment on what they think might occur in the mesoderm, are similar pools present here as well?
4. As the authoirs state themselves, Rho does not only affect actin via diaphanous, but of course also myosin via Rock. So it would be good to refelect this more in the interpretation and discussion

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of data, as the causal timeline could be complex.

We thank the reviewer for reminding us to address this point and to discuss this excellent recent paper. It was previously shown in mesoderm cells that pulsed myosin contractions condense the medio-apical F-actin network, but that this is often followed by F-actin network remodeling and that total F-actin levels decrease during apical constriction (Mason et al., 2013). Furthermore, Rho-kinase inhibition in mesoderm cells significantly disrupts this network, but does not inhibit the rapid assembly and disassembly of apical F-actin cables, which could reflect elevated actin turnover (Mason et al., 2013; Jodoin et al., 2015). To address the reviewer's points, we 1) now include a paragraph in the Discussion to discuss the Dehapiot et al. paper (Comment 3) and the possible roles of various pools of F-actin and Rock/myosin in shaping the tissue (Comment 4) (lines 360-380), and 2) show the apical surface of mesoderm and ectoderm at this stage and also germband extension with both phalloidin and Utr::GFP labeling, in order to show the apical F-actin network (Fig. S2A,B). Our data show that the apical F- actin network and Dia staining are similar across the mesodermal-ectodermal boundary, which suggests that this is not the reason for the different behavior of these cells.

More specific comments to experiments and figures:

5. Reduction of junction function by alpha-catenin-RNAi: how strong is the reduction in catenin? Could they label a-catenin in fixed embryos? The authors conclude the original pre-constriction patterning of F-actin intensity is not dependent on intact junctions, but they show that the increase in F-actin in the mesodermal cells concomitant with apical constriction is in fact impaired in the RNAi. Thus, the authors can also not conclude whether the continued accumulation of myosin and its persistence depend on intact junctions. The initial set-up of the myosin gradient in terms of intensity distribution is unaffected, but clearly dynamics, subcellular pattern, interconnectivity etc. of myosin are affected and thus may well depend on some mechanical feed-back. I find this section of the manuscript slightly overstated and feel the conclusion should be more cautious.

We thank the reviewer for pointing this out; we completely agree that we should have been more precise with our language. Our main conclusion was that myosin accumulation in a gradient does not require 'sustained mechanical connectivity'. We felt it was important, given our model of transcriptional patterning, to show that some patterns did not result from mechanics or even apical constriction. Alpha-catenin knock- down provides the cleanest and most severe disruption of cell adhesion that we can accomplish at this developmental stage. We showed that alpha-catenin-RNAi resulted in:
a) almost no intercellular connectivity in myosin structures (Yevick et al., 2019), and b) no apical constriction (this study, Fig. 3B). Others have also shown the effectiveness of alpha-catenin-RNAi in uncoupling apical actomyosin from junctions, which we have also cited (Fernandez-Gonzalez and Zallen, 2011; Levayer and Lecuit, 2013).
We: 1) revised the text in this section, clarifying that we are showing that specifically, the myosin gradient does not require adherens junctions, 2) included data better showing the depletion of junctional alpha-catenin in the alpha-catenin knockdown (alpha-catenin staining) and its effects on junctions and actomyosin (Fig. S4B-D).

6. Figure 1 versus Figure 2:
Why do the Utrophin-ABD virtual cross-sections look so fuzzy and bad in comparison to phalloidin labelled F-actin in the virtual cross-section in Fig. 1B and C? The labelling shown in 2B and D does not even look very junctional...

We apologize that we did not explain the difference in visualization methods more clearly. For live images (Figure 2), we used a projection of cross-sections, which includes 20 μm length along the anterior-posterior (AP) axis. This projection method is less dependent on the specific AP position of the cross-section and the specific cells being shown. We did this because the projection helps to visualize the tissue pattern in live images where fluorescence images are noisier than fixed images, which exhibit cleaner labeling (Fig. 1). To address this point, we have: 1) edited the text to make the method of visualization clearer, and 2) added fixed *snail* mutant embryo cross-sections that show the absence of F-actin depletion (Fig. S3B). Given that, a) Reviewer #3 specifically had questions about the snail mutant, b) live imaging was the

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best way to identify twist mutants unambiguously, and c) the mesoderm-ectoderm boundary in F- actin levels was clearly observed by live imaging, we did not include cross-sections of twist mutants.

7. Figure 5 C and D: the control gradients for myosin shown in C and D are completely different, for C the half-way height cell row is deduced as 5 whereas the (in theory identical) control measure in D has row 3 at halfway height! Why is this? Putting all curves together in the same panel would suggest that that C control curve is very similar to RhoGEF2-OE! This can't be right.

The reason for the different width of the gradients in these controls is the Sqh::GFP copy number. All of our experiments perturbing Rho were carefully controlled so as to ensure the same copy number of the fluorescent marker that we were visualizing. For technical reasons, we were only able to get 1 copy of the Sqh::GFP into the RhoGEF2-OE background. Having two copies of the Sqh::GFP appears to have a slightly activating effect; in fact, the reviewer might have noticed that ventral furrows with 2 copies Sqh::GFP (and a wider gradient) have lower curvature, consistent with our main conclusion (Fig. 7 C). The effects of fluorescently tagged markers were a concern for us and so we were careful to show that the effects of changing RhoA activity on tissue curvature occur regardless of the fluorescent marker (i.e., Sqh::GFP or Utr::GFP, Fig. 7 and Fig. S8).

8. Still in Figure 5: Panels C and D again, but for apical area: are the control and C-GAP-RNAi or RhoGEF2-OE curves significantly different? What statistics were used on this?

We thank the reviewer for this point. To address the reviewer's point, we have added myosin intensity as a function of physical distance, which showed a statistically significant difference in myosin width between both C-GAP-RNAi and RhoGEF2-OE and the genotype matched controls (Fig. 6C and D). Because cell-based analysis requires substantially more complex segmentation for each embryo and we did not have > 5 embryos per condition that were perfect for cell segmentation, we were unable to present statistical analysis on this, but present the data in Fig. S7E and F, which is consistent with the quantification of physical distance.

9. Supplemental Figure 1:
Panels in D: I appreciate this control, but would really also like to see the same control at a stage when folding has commenced and stretched cells are present at the margin of the mesoderm. How homogenous does the GAP43 label look in those?

We have added an apical projection of the same embryo, in which folding has already commenced, to Figure S1D, so its stage is clearer.

10. Supplemental Figure 5:
Panel 5 B: the authors conclude that the myosin gradient under RhoGEF2 RNAi is not smaller, but looking at the curves it in fact looks wilder. They also mention that the overall level of myosin in this condition is lower than the control...

We now include quantification of absolute levels in Fig. S6F to compare overall levels for all RhoA regulator disruptions. Based on these results, we have updated the language in lines 271-274 to reflect the effects of RhoGEF2 RNAi under the conditions used in this experiment more accurately.
We also statistically compared RhoGEF2 RNAi and control gradients and found no significant difference between these groups (Fig. 6D).

11. Following on from the above, a comment of Figure 7:
- The authors use RhoGEF2 RNAi stating that it affects the actin pattern, but the myosin pattern also seems affected. In line 318 the authors state that they use this condition to look at how junctional actin density affects curvature. I find this phrase misleading as It might lead the readers to think that RHoGEF2 RNAi only affects junctional F-actin, although it also affects myosin patterning.

We thank the reviewer for catching this, that's a good point. The maternal GAL4 drivers used

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in these two experiments were different, so we have revised the text in lines 271- 274, 285-291 and 326-331 to more accurately describe the experimental difference and the effect of RhoGEF2-RNAi on actin, myosin, and curvature.

- Line 311: confusingly, the authors state that an increase in the actomyosin gradient affects curvature. But it is only the myosin gradient that is increased, while the junctional actin gradient is flatter than the control in both C-GAP RNAi and RhoGEF2 OE (the distinction is even made by authors line 243). Could this be clarified?

We thank the reviewer for pointing out this imprecision on our part and have revised the language in line 299 and throughout the manuscript to more precisely describe the individual effects on myosin and F-actin pattern changes upon RhoA perturbation.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Mesoderm invagination during Drosophila gastrulation has been a paradigm for how regionally restricted gene expression locally activates Rho signalling and for how subsequently activated acto-myosin drives cell shape changes which in turn lead to a change in tissue morphology.
Despite the numerous studies on this subject and a good understanding of the overall process, several important aspects have remained elusive so far. Among these is the dynamics of cortical and junctional F-actin and its contribution to the shape changes of cells and tissue.
Previous studies have focused on MyoII, the „active" component of the actin cytoskeleton. The dynamics of the „passive" counterpart, namely actin filaments, has been neglected, although it is clear that Rho signalling controls both branches.

We thank the reviewer for carefully reading the manuscript. The reviewer raises important points that, even if not all feasible to address experimentally, can be addressed by being more precise with our language and conclusions.

1. Although I clearly acknowledge the efforts taken by the authors to define a function of cortical (junctional) F-actin in apical constriction and furrow formation, several central aspects of the study are not sufficiently resolved and conclusive. Rho signalling controls MyoII via Rok and F- actin via forming/dia, among other less defined targets. The role of MyoII and cortical contraction could be conclusively sorted out, since inhibition of Rok affects the MyoII branch but not the other branches. A similar approach, i. e. a specific inhibition/depletion without affecting the other branch, has not been taken yet for the F-actin branch. The authors have not resolved this issue. When analysing the mutants, the authors cannot distinguish the effect of Rho signalling on the MyoII and F-actin branch. For this reason the changes in F-actin distribution in the mutants are linked to changes in Myo activity and thus a function cannot be assigned to F- actin. In order to derive a specific role of F-actin distribution for furrow formation, the authors need to find experimental ways to affect F-actin levels without affecting MyoII, for example by analysing mutants for dia or other formins.

The reviewer's assertion that Rok and Diaphanous only affect myosin and actin, respectively, is oversimplified. Myosin binds actin and plays a critical role in organizing F-actin meshworks (Lehtimäki et al., 2021; Verkhovsky et al., 1995) and actin assembly likewise influences myosin localization and function (Reymann et al., Science, 2008) – actin and myosin are essentially a functional unit. In addition, both actin and myosin are required for adherens junction organization, providing another way in which both these cytoskeletal components are interconnected (Lecuit and Yap, 2015). Because RhoA coordinates actin assembly with myosin activation, we focused on carefully analyzing perturbations that affect RhoA's activation pattern.

We have put much effort into trying to modulate the F-actin pattern independently of myosin and have found that this is currently unfeasible for the following reasons: 1) *diaphanous* mutants and actin drugs affect RhoA signaling (Munjal et al., 2015; Coravos et al., 2016; Michaux et al., 2018), 2) *diaphanous* mutants and other gene depletions that lower actin turnover disrupt adherens junctions and tissue integrity (Homem et al, 2008; Mason et al., 2013; Jodoin et al., 2015; Levayer et al., 2011), and 3) Actin disruption and *diaphanous* mutants have severe cellularization defects (Afshar et al., 2000).

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Likewise, we found it is not feasible to conclusively sort out the role of MyoII with Rok inhibitor. We attempted to analyze tissue-level actomyosin patterns in Rok inhibitor- injected embryos and, similar to diaphanous depletion, Rok inhibitor injection led to complex disruptions of tissue organization. Indeed, we and others have shown: 1) Rok inhibitor disrupts apical F-actin organization (Mason et al., 2013), and 2) disrupts adherens junction organization (Weng and Wieschaus, JCB, 2016; Coravos and Martin, 2016).

To address the reviewer's comment, we 1) examined Diaphanous localization and show that Dia does not have a clear tissue pattern across the mesoderm-ectoderm boundary (Fig. S2C), 2) added data showing lateral myosin accumulation in midline cells together with F-actin, consistent with a previous study (Gracia et al., 2019); again arguing that F- actin and myosin are functioning together (Fig. S2A), and 3) added a section to the Discussion (lines 369-380) explaining the difficulty in disentangling myosin, F-actin, and adherens junctions.

2. The authors employ a discontinuous spatial axis by the cell number. Although there are good arguments to understand and treat the cells as units, there are also good arguments for using a scale with absolute distance. I have doubts that the graded distributions presented by the authors are a result of this scaling with cell units. When looking at panel B of Fig 1 or Fig. 2A,B, for example, a sharp step like distribution is visible at the boundary between mesoderm and ectoderm anlage. In contrast a F-actin intensity distribution is graded after quantification. The graded distribution appears not to be a consequence of averaging because an even sharper step is very obvious in a projection along the embryonic axis as shown in panel B and D of Fig. 2, for example. The difference of a sharp step in the images and graded distribution after quantification with a spatial axis in cell number, is obvious for a-catenin in Fig. 3D and Rho signalling in Fig. 4 B. As the authors base their central conclusion (see headline) on the graded distribution, resolving the issue of spatial scale is a prerequisite of publication.

We thank the reviewer for their point. It was an excellent idea and we have included representative plots with a continuous spatial scale in addition to our cell-based analysis (see below, each trace is average line intensity for 1 embryo). The spatially resolved analysis shows similar patterns for F-actin, myosin and RhoA pathway components as the cell-based metric and we now include this data as Supplemental Fig. 4E and 5A.
In addition, we analyzed the effects of RhoA disruptions on gradient width for both cell- based and absolute distance scales, showing quantitative differences in both cases (Figure 6, S7).

3. The authors put the spatial distribution of Rho signalling and F-actin into the center of their conclusion. They do so by affecting the pattern with mutants in twist/snail and varying upstream factors of Rho signalling. With respect to myo activation this have been done previously although possibly with less detail and it is no new insight that the width of the mesoderm anlage and corresponding Rho signalling domain has a consequence on the shape of the groove and furrow. To maintain the conclusion of the manuscript that spatially graded Rho signalling is contributes to tissue curvature, more convincing ways to change the pattern of Rho signalling are needed. Changing the balance of GEF and GAP shows the importance of Rho signalling and possibly signalling levels but not the contribution of its spatial distribution.

A strength of our study was that we were able to stably 'tune' Rho signaling pattern and then follow tissue shape at later stages to determine the connection between the two. We respectfully disagree with the statement that, "with respect to myosin activation this has been done previously". In past work, we expanded myosin activation drastically by modifying embryonic cell fate, including changes in dorsal cell fates (Heer et al. 2017; Chanet et al., 2017). Here, we directly manipulate RhoA signaling, maintaining the width of the mesoderm anlage (Fig. S6C).
A central conclusion of our study is that RhoA activation level determines the width of myosin activation within a normally sized mesoderm anlage, which has not been done before and is now highlighted in our Abstract. The genetic approach presented in the paper was the best way we found to manipulate the spatial pattern of myosin/actin in a stable manner that lasts through invagination. It is worth noting that this approach allowed us to carefully 'tune' the level of RhoA activation so as to avoid elevating RhoA levels to the point that it disrupts signaling polarity within the cell (Mason et al., 2016). In our hands, optogenetic manipulation

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of RhoA, which requires continuous optical input, was less robust because: a) 2D tissue flow precludes delivering a consistent level of activation to given cells over the time course of invagination, b) tissue folding (i.e. 3D deformation) dramatically alters how much light is delivered to the mesoderm cells.

To address the reviewer's point, we: 1) edited the Results to explicitly state that we did not alter the pattern of RhoA activation without altering RhoA signaling levels and (lines 258-263, 305 and 311-312), 2) include Snail stainings showing that the width of the mesoderm anlage is not altered by changes in RhoA signaling in Figure S6C so there is no confusion about this point, and 3) include a mechanical model in Figures 7 and S9 that compares how altering signaling levels vs. altering the spatial distribution of signaling separately affect fold curvature, respectively. This model demonstrates that widening the myosin gradient pattern and the domain of uniformly high myosin around the ventral midline can reduce curvature at the center of the fold, whereas increasing myosin contractility levels alone would increase central curvature.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Previous work has shown that mesoderm invagination at the ventral midline of the Drosophila embryo requires precise spatial regulation of actomyosin levels in order to fold the tissue. In this work, Denk-Lobnig and colleagues further investigate the spatial distribution of myosin and F- actin in the mesoderm and how these patterns are established. The authors identify an F-actin pattern at the apical cell junctions that emerges upon folding, with elevated levels in the cells around the ventral midline, a decrease in junctional F-actin in the marginal mesoderm, and then an increase at the mesoderm-ectoderm border. They identify Snail and Twist as regulating different aspects of establishing this F-actin pattern. Additionally, by modulating RhoA activity (downstream of Twist) the authors are able to alter the width of the actomyosin pattern without affecting the width of the mesoderm tissue, which in turn affects the curvature of the tissue fold and the post-fold lumen size.

The authors have conducted an elegant quantitative analysis of the distribution of actin, myosin and several of their regulators across the tissue. The study makes an attempt at integrating a large amount of information into a model of tissue folding, and the concept of mechanical gradients is exciting and still underexplored. I am concerned that the interpretation of some results focuses on specific details but ignores larger scale effects (e.g. potential effects of some of the manipulations on the ectoderm, and the impact that that could have on tissue folding are largely ignored). The statistical analysis of several results should also be improved. I suggest to address the following points.

We thank the reviewer for their interest in our work and their important suggestions.

MAJOR

1. Line 127 and Figure 1E: The authors argue that there is an anticorrelation between F-actin distribution and cell areas. However, an R-squared value of 0.1083 rather suggests little-to-no correlation. The authors should evaluate the statistical significance of that correlation.

To indicate whether the relationship between F-actin distribution and cell areas is significant, we added the results of an F-test for overall significance for our regression analysis, as well as sample size. The F-statistic for this analysis of 736 cells is F = 89.2, p- value = 4.7e-20.

2. Figure 5: claims that the width of the actomyosin gradient is affected by the various perturbations should be supported with statistical analysis. For example, the half-maximal gradient position for each individual myosin trace could be calculated (instead of using the mean trace), displayed using a box plot, and tested for significance using the Mann-Whitney U test, as in Figure 7. This is slightly complicated by the fact that the control group in Figure 5C is the same as the control group in Figure 3E, which needs to be carefully considered. Also, similar calculations should be made for the F-actin data in Fig 5E-G since throughout the rest of the paper, the authors refer to the width of the "actomyosin gradient" which implicates both myosin and actin.

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We thank the reviewer for this point. We now show box plots quantifying myosin and apical area gradient width in Fig. 6 and S7.
In order to meaningfully compare myosin gradients statistically, we decided compare the half-maximal width of average line profiles of myosin intensity in addition of the cell- based quantitative analysis in Figure 6. This allowed us to achieve bigger sample sizes and include a bigger proportion of our imaging data sets in analysis. To allow for multiple comparisons using the same control, we used Kruskal-Wallis testing when applicable, which is analogous to one-way ANOVA for non-parametric data, and a post- hoc test to determine which pairs have significantly different distributions. Other pairwise comparisons were done with the Mann-Whitney U test. Our results in Fig. 6C and D show that both the half maximal width of RhoGEF2 O/E and C-GAP RNAi embryos are significantly higher than their respective controls.
As our main conclusion for F-actin is that F-actin depletion levels are changed by RhoA in marginal mesoderm cells, we compared F-actin depletion in the marginal mesoderm. We have updated the language throughout the manuscript to distinguish between actin and myosin patterns and the aspects of the patterns that we are comparing. A box-and-whisker plot and statistical comparisons of F-actin depletion relative to the ectoderm are shown in Fig. 6 I. In order to make meaningful statistical comparisons, we increased the number of embryos analyzed in this experiment during revision. Our data demonstrates that RhoGEF2 O/E has significantly more F-actin density in marginal mesoderm cells than controls. C-GAP RNAi was not significantly different, consistent with the lesser effect on RhoA activation of this perturbation (Fig. S6F).
RhoGEF2-RNAi embryos have increased average depletion, which was not statistically significant, but consistent with the effect of RhoGEF2 OE.

3. Line 142 and Figure 2B-C: I was confused by the description of the snail phenotype:
- a. the claim that in snail mutants actin levels are uniform: based on Figure 2C, I'd say that F-actin levels decrease across the mesoderm moving away from the ventral midline, and that the main issue is with the accumulation of actin in the distal end of the mesoderm. The authors should better justify the claim that F-actin levels are uniform in snail mutants (or remove it).
Maybe comparing F-actin levels in the first four or five rows of the mesoderm?
- b. how about the increase of F-actin in the distal mesoderm, just adjacent to the ectoderm boundary? Why is it gone in snail mutants?

a. We agree that the intensity in all embryos appears to decrease on the sides of the embryos when imaged in this way, but it is also clear that there is no abrupt increase in F-actin density going into the ectoderm. In our experience, the edge effect is due to the distance of the side of the embryo from the coverslip rather than actual lower F-actin density. This is suggested by: a) the fact that all *snail* mutant embryos peak at the center of the image even though they are not all oriented with the ventral side perfectly on top, and b) all embryos exhibit an intensity decrease within the ectoderm toward the edges of the image that are further away from the coverslip (Fig. 2 C, E, F). We have: 1) updated the text to include an explanation, and 2) provided fixed and stained *snail* mutant cross- sections without this effect of imaging depth for comparison, in Fig. S3B.
b. We show in Figure S1C that in wild-type, F-actin does not actually increase in cells at the ectoderm boundary, but merely decreases in lateral mesoderm cells. Thus, it is likely that snail mutant embryos are merely lacking patterning in the mesoderm, where snail is active.

4. With alpha-catenin-RNAi, F-actin depletion across the mesoderm still occurs, but junctional F-actin levels are not increased around the midline. While some explanations are offered in the text, the reason for this phenotype seems important for the story. The text in lines 204-205 suggests that F-actin that would normally be localized to the apical junctions is instead being drawn into medioapical actomyosin foci. Is this idea supported by evidence that medioapical F- actin in control embryos is lower than in alpha-catenin RNAi?

We appreciate the reviewer's suggestion to explain this more thoroughly. We find that in alpha-catenin-RNAi and even *arm* (ß-catenin) mutant embryos, junctional complexes (i.e., E-cadherin) are drawn into the myosin spot through continuous contractile flow (see Fig. S4B and Martin et al., 2010 for *arm*). To make this clear, we now include data showing 1) the effects of alpha-catenin RNAi on F-actin and E-cadherin localization in fixed embryos (Fig. S4B and C),

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and 2) apical F-actin accumulation of UtrGFP-labeled alpha-catenin RNAi embryos (Fig. S4D).

5. Figure 6A: there is a correlation between cell position and the productivity of myosin pulses, which the authors attribute to the RhoA gradient. This should be more definitively demonstrated by:

- a. Plot and calculate the correlation between RhoA levels (measured with the RhoA probe) and the change in cell area caused by a contraction pulse. Is this a significant correlation?
- b. How does myosin persistence change when RhoA is manipulated, e.g. in RhoA overexpressing embryos or in RhoA RNAi?

It has already been shown that there is a correlation between myosin amplitude and apical constriction amplitude (Xie et al., 2015). Apical myosin and Rho-kinase localization depends entirely on RhoA activity (Mason et al., 2016) and Rho-kinase co-localizes precisely with myosin in both space and time (Vasquez et al., 2014). Changing levels of the RhoA regulator C-GAP has been shown to affect myosin persistence and the productivity of apical constriction, with higher C-GAP causing less productive constriction (Mason et al., 2016). However, understanding the effect of RhoA activity on subcellular myosin dynamics is complicated and beyond the scope of the present study. Therefore, we 1) moved the section on myosin pulsing to earlier in the paper so as to not imply that RhoA activity levels are causing the difference in area behaviors, and 2) have updated the text so as not to address how RhoA activity affects the behavior of myosin pulses. Our observation do show how cytoskeletal activity is patterned across the mesoderm, so we think it has value and that it should be included in this paper.

MINOR

1. The authors should indicate if the myosin shown in Figure 1A is junctional or medioapical. If it is junctional, does medioapical myosin better match junctional F-actin and cell areas? Similarly, if they are showing medioapical myosin, how does junctional myosin compare to junctional actin? It seems to me that consistently comparing the patterns of junctional F-actin and medioapical myosin (and RhoGEF2, RhoA, and ROCK in Figure 4) could be somewhat misleading, as the pools compared localize in different subcellular compartments.

The myosin images shown throughout the paper are medioapical myosin. Junctional myosin in mesoderm cells is lower in intensity and cannot easily be seen by live imaging. We agree that it is important for the reader to see all pools of these proteins. Therefore, we now include high resolution images of actin and myosin at both apical and subapical positions for midline mesoderm, marginal mesoderm, and ectoderm cells at the time of folding in Figure S2A, B. We also justify in lines 95-98, 104-111 why the analyzed pools were chosen, respectively.

2. Most of the intensity traces for myosin and F-actin are presented as normalized intensity, relative to the highest intensity in the trace. However, there are claims throughout the text about the relative levels of myosin (ex. Line 241) and F-actin (conclusions based on Fig. 2B-D) that should be supported by quantification. It seems that changes in intensity for both F-actin and myosin, in addition to shape of the gradient, would contribute to the understanding of actomyosin regulation in this tissue. However, if intensities cannot be directly compared between groups due to variation in imaging settings or staining protocols, there should be no claims made about changes in overall F-actin or myosin intensity.

We appreciate the point made by the reviewer here. To address this point, we have provided data for absolute levels in relevant cases in Figure S6F and the text in result sections corresponding to Figures 6 and 7 to be more precise in our conclusions.

3. The significance of the correlation in Figure 7E should be quantified.

We now report the p-value for the F-test for overall significance for our regression analysis of

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this data. The F-statistic for this analysis is F = 15.6, p-value = 0.00103.

4. Supplemental Figure 2: does the segmentation image match the second Z reslice immediately above? It does not appear so, or perhaps they are just not aligned. Having the two match would be more convincing of the segmentation technique.

We have updated this figure to ensure that matching images are used.

Reviewer #3 (Significance (Required)):

The authors have conducted an elegant quantitative analysis of the distribution of actin, myosin and several of their regulators across the tissue. The study makes an attempt at integrating a large amount of information into a model of tissue folding, and the concept of mechanical gradients is exciting and still underexplored. I am concerned that the interpretation of some results focuses on specific details but ignores larger scale effects (e.g. potential effects of some of the manipulations on the ectoderm, and the impact that that could have on tissue folding are largely ignored). The statistical analysis of several results should also be improved.

This is a great point. It is important to note that our conclusions required us to 'tune' the expression of GEF and the depletion of GAP with GAL4 drivers to get expression levels that do not dramatically affect RhoA polarity within mesoderm cells, but that alter the tissue level pattern within the mesoderm. Furthermore, we were cautious in making sure that our perturbations that elevate RhoA activation level did not lead to elevated myosin in the ectoderm (Fig. 5A and B). It is worth noting that RhoGEF2 is still full-length in all cases and has all of the normal regulatory domains that allow its activity to be restricted to the mesoderm at this stage via Gα signaling (De Las Bayonas et al., 2019; Aittaleb et al., 2010). To more explicitly show the effect of our perturbations on ectoderm cells, we now include higher resolution images comparing myosin and F-actin organization/levels in the ectoderm for our manipulations of RhoA signaling (Fig. S6A and B).

Second decision letter

MS ID#: DEVELOP/2020/199232
MS TITLE: Combinatorial patterns of graded RhoA activation and uniform F-actin depletion promote tissue curvature AUTHORS: Marlis Denk-Lobnig, Jan F Totz, Natalie C Heer, Jörn Dunkel, and Adam Martin
ARTICLE TYPE: Research Article

I have looked carefully at your responses to the referees' comments, which I found clear and convincing. In light of this I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Where referee reports on this version are available, they are appended below.

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