Mechanical competition alters the cellular interpretation of an endogenous genetic programme

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Revision 0

Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Bhide and colleagues present an insightful study of how cellular mechanics influences differential cell behaviour during morphogenesis despite apparent genetic homogeneity of the cellular ensembles. They dissect the extensively studied system of mesoderm invagination in Drosophila, focussing on the differences in cell behaviours between the cells in the

middle of the infolding tissue and on the periphery that, as far as we know, share a common gene expression profile. They describe sub-cellular dynamics of major effector of apical constriction morphogenesis, the myosin motor distribution, in the invaginating cells and conclude that differences in myosin levels alone cannot account for the observed differences in cell behaviours. In order to understand the cell behaviour inhomogeneity, they turn to biophysical simulation and in an impressively exhaustive manner substantiate the idea that non-linear effects are required for explaining the phenomenon. This theoretical treatment fits well with the notion that the genetic identity of the cells but rather cell-cell mechanical coupling determine the differences in invaginating cell's behaviours. Additionally, the modelling is consistent with the myosin asymmetry and dynamics in the cells whose behaviours is being contrasted. Complementary, and beautifully executed filament-based modelling of microscopic actomyosin contractility further corroborates this view. Finally, the proposed model of non-linear actomyosin contractility dynamics governing the differential cell behaviour across genetically homogenous cellular field, is challenged by two complementary laser ablation and optogenetic experimental approaches. Overall, the results represent convincing evidence that points the tissue mechanics field of Drosophila mesoderm into an interesting new direction and has general implications for the understanding of the interplay between genetic regulation and emergent behaviours of cells operating in mechanically complex multicellular embryonic context.

The study is meticulously executed, highly quantitative and combines effectively experiment and theory. I have only minor comments that concern in particular the presentation of the results.

The paper is very dense and the text does not complement well the results presented in the main figures. Many panels in the Figures are not referred to explicitly. Figure elements are referenced out of order both within and across Figures. Sometimes, particularly, in the last two Figures (3 and 4) the reader is left alone to figure out what the data show (with the appropriately terse legends and without the clear narrative in the text, it is an uphill battle for non-specialists). Some key results are hidden in the sea of elements within the Figure 2 that contains the most important, relevant and impressive data. As an example, on line 168 the authors point to panel 2F to demonstrate the asymmetry of myosin distribution in some cells. To the best of my understanding, this phenomenon is actually shown in Fig 2E which is curiously not referenced at all.

Similarly, Figure 2K and L provide crucial data substantiating much of the conclusions of the paper. It requires a major effort to understand what the graphs mean.

The following simulation results are quite impressive and would deserve a separate Figure which could provide more space for explaining what the parameter maps actually show. What is for instance plotted on the Y axis as

steepness?

Secondly, I find the overall narrative of the manuscript needing some reorganisation. The main question is set-up extremely well, however in the middle of the manuscript the focus on the connection between cell behaviours and genetic programs is lost. New conclusions on force transmission between cells emerge, however they are not obviously connected with the question posed from the onset and addressed in the discussion section. My impression is that the authors are conservative in their reasoning, however it does compromise the overall message of the story that should ideally focus on one subject. I find the combined evidence presented sufficiently supportive of the model that is beautifully and eloquently presented in the concluding sentence of the paper:

"This mechanism, which we propose corresponds to the non-linear behaviour predicted by the models, would apply both to central and to lateral cells, with a catastrophic 'flip' being stochastic and rare in central cells, but reproducible in lateral cells because of the temporal and spatial gradient in which contractions occur."

This may not turn out to be the entire story or even entirely correct, but it is certainly and exciting way of thinking about the problem. I wish that the manuscript would stay more on this subject throughout and provide intermediate conclusions supporting this model as the story develops.

Few more minor comments:

Line 36 - typo

Line 97 - starting bracket missing

Line 126 - data on intensity are presented here. There is also a panel on concentration (Fig 1H). Where is this discussed?

Line 132 - panel 2G - disruptive out of sequence reference to a future figure

Line 135 - with this regard - please spell out this important conclusion

Line 183 - typo

Line 210 - insects do not have intermediate filaments

Line 238 - please provide a hint of how such global ablations are performed

Line 240 - walk us through the Figure, it is too complex to figure it out

alone

Line 245 - why is the clear hypothesis mentioned above (point 2 rephrased?

Line 273 - vague statement

3. Significance:

Significance (Required)

The results represent convincing evidence that points the tissue mechanics field of Drosophila mesoderm into an interesting new direction and has general implications for the understanding of the interplay between genetic regulation and emergent behaviours of cells operating in mechanically complex multicellular embryonic context.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Bhide and colleagues explore the mechanisms of cell expansion in epithelial morphogenesis. During the invagination of the Drosophila mesoderm, cells in the center of the prospective mesoderm constrict under the action of actomyosin pulses, while lateral cells elongate towards the center of the mesodermal placode to accommodate the reduction in apical surface of the central cells. Central and lateral cells display strong similarities in terms of gene expression. How are thus this different behaviors (contraction and expansion) accomplished? The authors found that both central and lateral cells assemble actomyosin networks, although lateral cells do it with a certain delay. Mathematical models of cell constriction across the mesoderm using different strain-stress responses showed that strain-induced cell softening was necessary recapitulate the patterns of constriction and expansion observed in vivo. Furthermore, modelling predicts that cells can stretch until the actin networks yield and break. Laser ablation and optogenetic reduction of contractility in central cells results in a reduction in the apical surface area of lateral cells. An optogenetic increase in contractility in lateral cells caused an increase in apical area in central cells. Together, these data suggest that mechanical cues can override and contribute to sculpting genetically defined morphogenetic domains.

I propose to address the following points before further considering the manuscript:

MAJOR

1. Figure 3: following laser ablation of central cells, lateral cells reduce their apical surface. How do the authors know that this reduction in lateral cell apical surface area is an active process, driven by actomyosin-based contraction, rather than a passive response to the expansion of the wound induced by laser ablation? A similar argument could explain the constriction of lateral cells after optogenetic inhibition of actomyosin networks: the central cells relax, expand and compress the lateral cells. To demonstrate active responses of the lateral cells upon laser ablation and optogenetic manipulations of central cells, at the very least the authors should show the distribution of myosin in the lateral cells that constrict and demonstrate the assembly of contractile networks.

2. Modelling suggests that actin networks yield and break in lateral cells. Does this occur in vivo?

3. Lines 166-175: The authors propose that constriction of a cell affects the localization of myosin in its neighbors. However, this is not directly measured. The authors should quantify the relative myosin offset in the cells around constricting cells, and show that that offset is greater (and oriented towards the constricting cell) than in cells around expanding cells. There should be a correlation between the relative size change of a cell and the myosin offset (not just concentration) in their neighbours. In addition, does optogenetic activation of constriction in lateral cells affect the offset of myosin networks in central cells?

4. Fig. 2E-F: the authors argue that the mean myosin concentration in lateral cells at certain times is equivalent to that of central cells earlier in the invagination process. However, the fraction of apical surface area covered by myosin network is consistently lower for lateral cells (and also for central cells that remain unconstricted!). Have the authors considered this fact, and if not, why? Wouldn't this explain, at least in part, why some cells constrict and others do not, if medial myosin networks drive the disassembly of the apical surface? If myosin activity were increased in laterals cells once central cells begin constricting, would that lead to an increased fraction of lateral cell surfaces covered by actomyosin networks and to reduced lateral cell elongation?

MINOR

1. Image panels are missing scale bars in many figures.

2. Fig. 1C'-D': The authors should include a color bar to provide some indication of the scale of the apical areas measured. Same comment for other figures in which apical area is color-coded.

3. Supp. Fig. 2E-F, G-H and Supp. Fig. 6: what is the difference between myosin intensity and myosin concentration? Junctional vs medial localization? Or summed vs mean pixel value? Please be specific, the difference between intensity and concentration is not clear.

4. Line 118: Supp. Fig. 2 does not have panels I and K.

5. Line 223: the authors reference data at 175 sec, but Supp. Fig. 6 does not show any images at that time point. They should be added or a different time point indicated.

TYPOS

1. Abstract: "[in a supracellular context" should be "in a supracellular context".

2. Line 145: should this be a reference to Supp. Fig. 5 instead of Supp. Fig. 4?

3. Line 166: I am not sure how Supp. Fig. 5 supports this statement. Is this the right figure reference? Should it be Supp. Fig. 4 instead?

4. Line 881: "representing on line" should be "representing one line".

OPTIONAL

Tony Harris' lab showed that the Arf-GEF Steppke antagonizes myosin and facilitates cell deformation at the leading edge of the embryonic epidermis during Drosophila dorsal closure (West et al., Curr Biol, 2017). Does Steppke localize to junctions in lateral but not central mesoderm cells? Does the pattern of Steppke localization in the mesoderm change with manipulations to the contractility of central cells?

3. Significance:

Significance (Required)

This is an interesting study, and one that makes uses of beautiful tools, including quantitative microscopy and image analysis, mathematical modeling and optogenetic manipulations. The prediction that embryonic cells display non-linear stress-strain responses is exciting, as linearity has been the predominant assumption so far. However, I find that model predictions are not well supported by the data, and that alternative interpretations of some results are possible. Additionally, the paper lacks insight into the molecular mechanisms that facilitate stretching (although that could be the subject of a follow-up study).

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

In this study, the authors explore potential mechanisms for why some cell constrict while other cells expand, despite similar intrinsic genetic programs, during Drosophila ventral furrow formation at the onset of gastrulation. The authors combine quantitative analyses of cell shapes and myosin levels from multiphoton confocal and Multi-View SPIM imaging, optogenetic and laser perturbation experiments, and mechanical models to argue that nonlinear mechanical interactions between cells are required to explain the cell behaviors. Based on microscopic models of the actomyosin cytoskeleton in the tissue the authors argue that the required nonlinear mechanical behavior is consistent with actomyosin network reorganization.

Major comments:

- Although the area of investigation is exciting and the results are interesting, unfortunately the quality of the results and comparison between experiment and modeling in the current version of the manuscript are not convincing. Although it is not clearly explained in the manuscript, the experimental results on cell shapes, myosin intensity, laser manipulation, optogenetic perturbations appear to be from a single embryo or small number of embryos for each experiment (Figures 1, 3, 4). The authors state that the cell stretching pattern "was best recapitulated by a superelastic response", but did not provide direct quantitative comparisons of the different mechanical models to the experimental data to clearly demonstrate this. Moreover, the local optogenetic myosin recruitment experiments in Figure 4 do not provide sufficient information on optogenetic tool recruitment, myosin localization, or cell behaviors to justify the claim that the central cells are not activated by the optogenetic perturbation and are only responding to the forces from neighboring cells.

- The authors should provide direct quantitative comparisons of the models and experiments to clearly demonstrate their claims that the superelastic model is better than the linear model or other nonlinear models.

- The authors should do additional experiments and/or provide more details for the existing experiments (to include several embryos per condition) on myosin quantification, photo-manipulation, and optogenetics experiments. Additional controls would like be necessary for claims resulting from the optogenetics experiments in Figure 4.

- The additional time and resources required to address these concerns would depend on the experimental details, N values, and statistics in the current studies, which unfortunately were not described in the current manuscript.

- Methods descriptions for reproducibility are generally adequate, with the exception of N values and statistics - see above.

- Are the experiments adequately replicated and statistical analysis adequate? No, see above.

Minor comments:

1) Scale bars for images are missing throughout.

2) Number of embryos and cells analyzed missing throughout text and figure legends.

3) Units are missing for many quantities in figures and tables throughout.

4) Many figure references in the main text are incorrect, pointing either to the wrong figure or wrong figure panel.

5) Line 728. What time point was used for myosin concentrations used in the model? How might myosin dynamics influence these findings?

6) The authors show a few examples of myosin pulsing in lateral cells and then conclude that myosin pulsing is not qualitatively different from central cells (lines 135-136). The author should quantify the number of pulsing lateral cells as well as period and amplitude of pulsing, or discuss relevant results from prior studies in more detail to justify this conclusion.

7) Lines 145-150. The authors very briefly describe the results of the linear-stress strain response and conclude this did not yield outputs corresponding to in vivo data and leave this largely to the supplementary figures. This is a key point in the paper and deserves much more discussion and space in the main text. As mentioned in main comments above, a quantitative comparison of the different mechanical models to show that the superelastic model better describes the observations should be included (potentially as an inset to Fig 2D showing a quantitative measure of the quality of model fit to the data).

8) Lines 162-163. Provide more rationale for why strain-softening would most likely manifest as permanent or reversible cytoskeletal reorganization.

9) Lines 187-188. "This shows that forces acting on each cell from its neighbors have an important role in determining the cell's behavior." This seems somewhat obvious; perhaps a bit more explanation would help the reader to understand the importance of these results.

10) Lines 196-198. How were the concentrations and lengths of F-actin chosen? How were the concentration and properties of linkers chosen? How sensitive are the results to these details of the cytoskeletal composition?

11) Lines 238-244. It would be helpful to include some additional quantification that clearly shows the reader the differences in cell behaviors in control and perturbed tissue. For the optogenetics experiment, it would be important to show quantification that the lateral cells are not being directly perturbed during photoactivation of neighboring cells (e.g. due to light leakage). In both perturbations, it would be helpful to quantify how many cells in rows 7 and 8 constricted and by how much did they constrict? How reproducible were these effects?

12) Lines 245-252. A key assumption in interpreting this experiment seems to be that the central cells are not directly perturbed by the optogenetic activation. Additional quantifications of RhoGEF2-CRY2 and/or myosin should be shown to support this. It would be helpful to include some additional quantification that clearly shows the reader the differences in cell behaviors in control and experimental regions. How reproducible were these effects?

13) A section on statistics is missing from the methods section.

14) Line 615. Ensure that Eq. 1 is dimensionally consistent; crucially, what units are used for 'M'? If the model is non-dimensionalized, provide the reference scales.

15) Line 675: The investigated stress-strain relationships are presented in Table S1. What are the definitions of xpl and xsh?

16) Line 678: Parameter values for the stress-strain relationships are given in Table S2. Can you provide more information on how these values were selected and their units? How sensitive are the results to changes in these values? Provide references when possible.

17) Line 697. Please comment on why the embryo appears skewed to the right.

18) Line 712. A color-bar corresponding to this color-code is missing in the figure.

19) Lines 715-717. It seems panels E and E' are swapped in the legend.

20) Line 724 (Fig 2). It is difficult to read anything in panel K inset or Panel L inset.

21) Line 728. What does "embryo 1" refer to?

22) Line 732. A quantitative measure of the quality of the fits of the models to the experimental data should be included.

23) Line 739. What exactly does "Embryo 2" refer to?

24) Line 779. Why is a z-plane of 15 microns below surface chosen?

25) Line 797. Why is a z-plane of 25 microns below the surface chosen?

26) Line 900. Panel G in Supp Fig 5 is not described in figure description.

- Are prior studies referenced appropriately? Yes.

- Are the text and figures clear and accurate? No (see details listed above).

- It would be very helpful to the reader to show direct quantitative

comparison of the different mechanical models with the experimental observations to show how much better the nonlinear model is compared to the linear model. An extended explanation of experiments and experimental results within the main text would improve the manuscript.

3. Significance:

Significance (Required)

The key advance in this work is in identifying a potential role of nonlinear mechanical properties in contributing to distinct cell behaviors within a tissue during development in vivo. This contributes to a growing body of work highlighting the importance of cell and tissue mechanical properties in regulating cell behaviors during the formation of tissue structure.

This work adds to a growing body of work connecting actomyosin contractility in cells to tissue-scale behavior during development. This work provides a unique mechanical modeling perspective to the study of apical constriction during Drosophila ventral furrow invagination, highlighting a potential role for superelastic cell mechanical behaviors during morphogenesis in vivo.

The finding would be of interest to researchers working in the areas of morphogenesis, mechanobiology, the cytoskeleton, and active matter.

This reviewer's expertise is in experimental studies of the cytoskeleton and cell mechanics during morphogenesis.

Response to referees
(our responses in green font)

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

Bhide and colleagues present an insightful study of how cellular mechanics influences differential cell behaviour during morphogenesis despite apparent genetic homogeneity of the cellular ensembles. They dissect the extensively studied system of mesoderm invagination in Drosophila, focussing on the differences in cell behaviours between the cells in the middle of the infolding tissue and on the periphery that, as far as we know, share a common gene expression profile. They describe sub-cellular dynamics of major effector of apical constriction morphogenesis, the myosin motor distribution, in the invaginating cells and conclude that differences in myosin levels alone cannot account for the observed differences in cell behaviours. In order to understand the cell behaviour inhomogeneity, they turn to biophysical simulation and in an impressively exhaustive manner substantiate the idea that non-linear effects are required for explaining the phenomenon. This theoretical treatment fits well with the notion that the genetic identity of the cells but rather cell-cell mechanical coupling determine the differences in invaginating cell's behaviours. Additionally, the modelling is consistent with the myosin asymmetry and dynamics in the cells whose behaviours is being contrasted. Complementary, and beautifully executed filament-based modelling of microscopic actomyosin contractility further corroborates this view. Finally, the proposed model of non-linear actomyosin contractility dynamics governing the differential cell behaviour across genetically homogenous cellular field, is challenged by two complementary laser ablation and optogenetic experimental approaches. Overall, the results represent convincing evidence that points the tissue mechanics field of Drosophila mesoderm into an interesting new direction and has general implications for the understanding of the interplay between genetic regulation and emergent behaviours of cells operating in mechanically complex multicellular embryonic context.

The study is meticulously executed, highly quantitative and combines effectively experiment and theory. I have only minor comments that concern in particular the presentation of the results.

The paper is very dense and the text does not complement well the results presented in the main figures. Many panels in the Figures are not referred to explicitly. Figure elements are referenced out of order both within and across Figures. Sometimes, particularly, in the last two Figures (3 and 4) the reader is left alone to figure out what the data show (with the appropriately terse legends and without the clear narrative in the text, it is an uphill battle for non-specialists). Some key results are hidden in the sea of elements within the Figure 2 that contains the most important, relevant and impressive data.
We have split this figure in two, moved some of the results from Suppl. Fig. 5 into one of its parts and included new calculations and data. We have also extended the description of these results in the main text and in the figure legends.

As an example, on line 168 the authors point to panel 2F to demonstrate the asymmetry of myosin distribution in some cells. To the best of my understanding, this phenomenon is actually shown in Fig 2E which is curiously not referenced at all.

51 *We have corrected the references to the panels*
52
53 Similarly, Figure 2K and L provide crucial data substantiating much of the conclusions
54 of the paper. It requires a major effort to understand what the graphs mean.
55 The following simulation results are quite impressive and would deserve a separate
56 Figure which could provide more space for explaining what the parameter maps
57 actually show. What is for instance plotted on the Y axis as steepness?
58 *We have added the following explanation: "The 'width' of the profile is the number of*
59 *cells with maximum value; the 'steepness' is the slope between minimal and maximal*
60 *values (equation 2 in materials and methods)."*
61
62 Secondly, I find the overall narrative of the manuscript needing some reorganisation.
63 The main question is set-up extremely well, however in the middle of the manuscript
64 the focus on the connection between cell behaviours and genetic programs is lost. New
65 conclusions on force transmission between cells emerge, however they are not
66 obviously connected with the question posed from the onset and addressed in the
67 discussion section.
68 *To us, the section on force transmission seemed like an important component of the*
69 *issue of intrinsic versus extrinsically determined cell behaviours. We had seen that the*
70 *intrinsic programme of the cells, as reflected in their myosin levels, might not be*
71 *sufficient to explain the difference between stretching and constricting. If their*
72 *behaviour is not intrinsically determined, then there must be something acting from the*
73 *outside, and we are looking here at what that might be, i.e. we need to find out how the*
74 *potential constriction is influenced. The first model tests under which conditions*
75 *differential contractility leads to different 'cell' behaviours. This in turn leads directly to*
76 *the question of the forces the cells in the epithelium exert on each other.*
77
78 My impression is that the authors are conservative in their reasoning, however it does
79 compromise the overall message of the story that should ideally focus on one subject. I
80 find the combined evidence presented sufficiently supportive of the model that is
81 beautifully and eloquently presented in the concluding sentence of the paper:
82
83 "This mechanism, which we propose corresponds to the non-linear behaviour predicted
84 by the models, would apply both to central and to lateral cells, with a catastrophic 'flip'
85 being stochastic and rare in central cells, but reproducible in lateral cells because of
86 the temporal and spatial gradient in which contractions occur."
87
88 This may not turn out to be the entire story or even entirely correct, but it is certainly
89 and exciting way of thinking about the problem. I wish that the manuscript would stay
90 more on this subject throughout and provide intermediate conclusions supporting this
91 model as the story develops.
92
93 Few more minor comments:
94 *We have corrected all of the typos, mistakes and omissions and adapted the text, as*
95 *mentioned below.*
96
97 Line 36 - typo
98 Line 97 - starting bracket missing
99 Line 126 - data on intensity are presented here. There is also a panel on concentration
100 (Fig 1H). Where is this discussed?

101 *An explanation (definition) has been added to the main text.*
102 Line 132 - panel 2G - disruptive out of sequence reference to a future figure
103 Line 135 - with this regard - please spell out this important conclusion
104 *We have expanded this part, basically introducing the conclusion more clearly (we*
105 *hope).*
106 Line 183 - typo
107 Line 210 - insects do not have intermediate filaments
108 *We have added 'mammalian' to the reported experiment in the text, to make it clear*
109 *that this does not refer to Drosophila cells*
110 Line 238 - please provide a hint of how such global ablations are performed
111 *We have added this – both explicitly, and the relevant references.*
112 Line 240 - walk us through the Figure, it is too complex to figure it out alone
113 *We have added a more extensive explanation both in the text and in the new figure*
114 *legend.*
115
116 Line 245 - why is the clear hypothesis mentioned above (point 2) rephrased?
117 Line 273 - vague statement
118 *We have changed the text in response to these useful pointers.*
119
120 Reviewer #1 (Significance (Required)):
121
122 The results represent convincing evidence that points the tissue mechanics field of
123 Drosophila mesoderm into an interesting new direction and has general implications for
124 the understanding of the interplay between genetic regulation and emergent
125 behaviours of cells operating in mechanically complex multicellular embryonic context.
126
127
128 Reviewer #2 (Evidence, reproducibility and clarity (Required)):
129
130 Bhide and colleagues explore the mechanisms of cell expansion in epithelial
131 morphogenesis. During the invagination of the Drosophila mesoderm, cells in the
132 center of the prospective mesoderm constrict under the action of actomyosin pulses,
133 while lateral cells elongate towards the center of the mesodermal placode to
134 accommodate the reduction in apical surface of the central cells. Central and lateral
135 cells display strong similarities in terms of gene expression. How are thus this different
136 behaviors (contraction and expansion) accomplished? The authors found that both
137 central and lateral cells assemble actomyosin networks, although lateral cells do it with
138 a certain delay. Mathematical models of cell constriction across the mesoderm using
139 different strain-stress responses showed that strain-induced cell softening was
140 necessary recapitulate the patterns of constriction and expansion observed in vivo.
141 Furthermore, modelling predicts that cells can stretch until the actin networks yield and
142 break. Laser ablation and optogenetic reduction of contractility in central cells results in
143 a reduction in the apical surface area of lateral cells. An optogenetic increase in
144 contractility in lateral cells caused an increase in apical area in central cells. Together,
145 these data suggest that mechanical cues can override and contribute to sculpting
146 genetically defined morphogenetic domains.
147
148 I propose to address the following points before further considering the manuscript:
149
150 **MAJOR**

151
152 1. Figure 3: following laser ablation of central cells, lateral cells reduce their apical
153 surface. How do the authors know that this reduction in lateral cell apical surface area
154 is an active process, driven by actomyosin-based contraction, rather than a passive
155 response to the expansion of the wound induced by laser ablation?
156 A similar argument could explain the constriction of lateral cells after optogenetic
157 inhibition of actomyosin networks: the central cells relax, expand and compress the
158 lateral cells.
159 *With regard to the comparison to wounds, it is important to note that the epithelium is*
160 *not actually wounded by either ablation method. Thus, while the treatments ablate the*
161 *actyomyosin meshwork, they do not ablate or kill the cells. Perhaps the term is an*
162 *unfortunate choice, since it is more commonly used in developmental biology for killing*
163 *cells. However, here the cells remain intact and when the optogenetic or laser*
164 *treatment is released the cells resume their physiological activities.*
165 *We have added a note in the text and now refer to 'laser microdissection', a term of art*
166 *in the field, for more clarity.*
167 *Regarding the more important question of what is the active process, expansion of the*
168 *central cells or constriction of the lateral cells, a contribution from expanding central*
169 *cells is of course in theory not impossible.*
170 *However, for this scenario to work, in the absence of pulling from the lateral cells, there*
171 *would have to be a force that is generated in the central cells, in this case a pushing*
172 *force that would expand the cells and act on the lateral cells. We have shown in our*
173 *previous work that if the actomyosin is dissected in dorsal cells, which are not*
174 *surrounded by potentially contractile cells, the cells do not expand (Rauzi et al, 2017).*
175 *This shows that 'relaxing' by itself does not have 'expansion' as a consequence.*
176 *One would therefore have to consider how such a pushing force could arise in these*
177 *cells. We can think of only two possibilities: hydrostatic pressure or an active force from*
178 *the subcellular molecular machinery.*
179 *Considering hydrostatic pressure, if the apical actomyosin that is ablated was*
180 *responsible for maintaining such a pressure inside the cell (a reasonable assumption),*
181 *then releasing the actomyosin would allow the cell volume to push against the*
182 *neighbouring cell. However, such a recoil would occur on a very short time scale*
183 *(seconds), whereas we see the contraction of the lateral cells continuing over extended*
184 *periods (minutes).*
185 *Alternatively, expansive forces could be generated by the cytoskeleton. Cytoskeletal*
186 *pushing forces can come from microtubules (classical example: mitotic spindle;*
187 *epithelial morphogenesis: work from T. Harris and B. Baum labs: PMID 18508861 and*
188 *20647372), or from continuous creation of new cross-linked or branching actin*
189 *networks pushing against plasma membranes, as in the leading edge of crawling cells.*
190 *But the microtubules in the blastoderm cells are not oriented in such a way they could*
191 *provide a force in the correct dimension in these cells (the majority is oriented along the*
192 *apical-basal axis). In addition, the connection between MT and the plasma membrane*
193 *depends on the cortical actin meshwork (involving, for example, the actin-binding*
194 *proteins P120-Catenin or patronin/Shot; Roeper lab, PMID 24914560, StJohnston Lab,*
195 *PMID: 27404359) but the connection of actin with the plasma membrane has been*
196 *severed in the optogenetically manipulated cells.*
197 *By contrast, we show that normal lateral mesodermal cells possess a contractile actin*
198 *network. So the only sustained force generated in the system at this point is the*
199 *contractile force in lateral cells (which is normally counteracted by the stronger*
200 *contractile force from central cells).*

201 *Thus, we conclude that the expansion of central cells is a passive response to a*
202 *contractile force from lateral cells, not an active process and conversely, the*
203 *constriction of lateral cells is an active autonomous process.*
204
205 To demonstrate active responses of the lateral cells upon laser ablation and
206 optogenetic manipulations of central cells, at the very least the authors should show
207 the distribution of myosin in the lateral cells that constrict and demonstrate the
208 assembly of contractile networks.
209 *We have now included the requested data for the experiments with laser ablations.*
210 *Suppl. Fig. 8 and Suppl. video 3 show the myosin that accumulates in lateral cells.*
211 *It would be nice also to be able to show this for the optogenetic experiments. However,*
212 *despite trying hard, we have not succeeded in generating healthy embryos that carry*
213 *the entire set of transgenes that are necessary to carry out the optogenetic*
214 *experiments and at the same time visualize myosin (see also response to referee 2,*
215 *point 3).*
216
217
218 *2. Modelling suggests that actin networks yield and break in lateral cells. Does this*
219 *occur in vivo?*
220 *We postulate that the skewed and inhomogeneous distribution of myosin and the large*
221 *myosin-free areas in stretched cells (lines 170 – 172 in the original text) are indications*
222 *of a yielding meshwork, or at least of uneven force distribution in the network that leads*
223 *to ineffective contraction or even release – i.e. functionally correspond to yielding.*
224 *We have made this more explicit now.*
225 *We have also added an additional panel quantifying more clearly the proportion of low-*
226 *myosin areas in lateral cells (now Fig. 3H).*
227 *Work from the Lecuit lab has recently shown beautifully that it is the connectivity of the*
228 *myosin mesh rather than the underlying actin meshwork that affects apical forces in*
229 *epithelial cells (PMID: 32483386), and our own findings are entirely consistent with*
230 *that.*
231
232 3. Lines 166-175: The authors propose that constriction of a cell affects the localization
233 of myosin in its neighbors. However, this is not directly measured. The authors should
234 quantify the relative myosin offset in the cells around constricting cells, and show that
235 that offset is greater (and oriented towards the constricting cell) than in cells around
236 expanding cells. There should be a correlation between the relative size change of a
237 cell and the myosin offset (not just concentration) in their neighbours.
238 *We now provide measurements of the rate of cell area change against the offset of*
239 *surrounding myosin (the distance of myosin from a cellular border). We see that*
240 *surrounding myosin is closer to the border of constricting cells and tends to be further*
241 *away from the borders of expanding cells.*
242 *We have added these data to the new Fig. 3I.*
243
244 In addition, does optogenetic activation of constriction in lateral cells affect the offset of
245 myosin networks in central cells?
246 *This is technically challenging. For such an experiment we would need an embryo to*
247 *express membrane and myosin markers in addition to the two optogenetic constructs*
248 *and the GAL4 driver. We tried multiple times to generate such a cross, but obtained*
249 *either no embryos or, at best, deformed embryos. We also tried to use the MCP-MS2*
250 *system in parallel to CRY2-RhoGEF2 but the crosses had the same problem. This*

251 *sensitivity to additional genetic load was also observed in the DeRenzis lab, who*
252 *generated these strains and tested and used them extensively.*
253
254 4. Fig. 2E-F: the authors argue that the mean myosin concentration in lateral cells at
255 certain times is equivalent to that of central cells earlier in the invagination process.
256 However, the fraction of apical surface area covered by myosin network is consistently
257 lower for lateral cells (and also for central cells that remain unconstricted!). Have the
258 authors considered this fact, and if not, why? Wouldn't this explain, at least in part, why
259 some cells constrict and others do not, if medial myosin networks drive the
260 disassembly of the apical surface?
261 *We believe in fact that this is precisely part of the picture and it was what we had*
262 *meant to propose, but the text was perhaps indeed just to condensed.*
263 *Thus, we had stated in line 169 of the original document:*
264 *"While the asymmetry is visible in all cell rows, <u>there are larger areas without</u>*
265 *<u>myosin</u> and the distance of displacement is greater in lateral cells (Fig. 2G-J)",*
266 *and in the discussion (line 277 – 285):*
267 *"Despite the homogeneous actin meshwork in stretching cells, the areas that are*
268 *free of active myosin occupy a large proportion of the apical surface – similar to*
269 *ectodermal or amnioserosa cells in which the connection of pulsatile foci to the*
270 *underlying actin meshwork is lost. ...*
271 *Dilution of cortical myosin may compromise a cell's ability to make sufficient physical*
272 *connections, in particular along the dorso-ventral axis, so that even if sufficient force is*
273 *generated, it cannot shorten the cell in the long dimension. In other words, even though*
274 *the cells have enough myosin to create force, the system is not properly engaged and*
275 *its force is not transmitted to the cell boundary."*
276 *However, we didn't state this with sufficient clarity in the results section and have*
277 *added an extra sentence to this effect.*
278
279 If myosin activity were increased in laterals cells once central cells begin constricting,
280 would that lead to an increased fraction of lateral cell surfaces covered by actomyosin
281 networks and to reduced lateral cell elongation?
282 *This is a really nice experiment, and we have indeed tried to induce activation at later*
283 *time points, but unfortunately this did not yield unambiguous results.*
284 *If we did the manipulation after the central cells had clearly constricted, then activating*
285 *lateral cells did not lead to their contraction. However, since this is a negative result*
286 *and we have no independent criterion for knowing how 'strong' the induced contraction*
287 *was (as explained above, we are unfortunately not able to visualize the myosin in these*
288 *experiments), and why it might not have been sufficient to overcome the pull from*
289 *central cells.*
290 *In this context it is worth remembering that in mutants in which myosin is overactivated*
291 *as a result of defective upstream signalling, lateral cells stretch less or not at all. See*
292 *PMID: 24026125 for gprk2 mutants and our own results for active Rho1:*
293

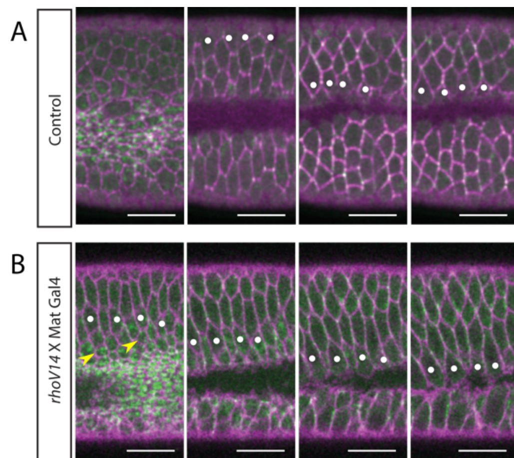

Figure: Confocal Z-section of embryos expressing sqh::GFP (myosin; green) and GAP43::mCherry (membrane; magenta) imaged ventrally. A constitutively active form of Rho1 is ectopically expressed using a maternal Gal4 driver, inducing activation of myosin in more lateral cells. White dots mark the mesectoderm determined by backtracing after ventral furrow invagination. Yellow arrows in B are constricted cells in row 7/8.

MINOR

1. Image panels are missing scale bars in many figures.
2. Fig. 1C'-D': The authors should include a color bar to provide some indication of the scale of the apical areas measured. Same comment for other figures in which apical area is color-coded.

We have added the missing elements

3. Supp. Fig. 2E-F, G-H and Supp. Fig. 6: what is the difference between myosin intensity and myosin concentration? Junctional vs medial localization? Or summed vs mean pixel value? Please be specific, the difference between intensity and concentration is not clear.

In the cases where we talk about myosin 'amount' we have now exchanged the term 'intensity', i.e the physical term for the amount of light, for 'amount' (i.e. that for which we use the light intensity as a proxy) and have explained in the main text how we define total apical myosin amount and apical myosin concentration (amount over area). However, in the cases where we are describing the actual image analysis, as in Suppl. Fig. 3, we use 'intensity' as the term of art that is used for the methods employed here. Similarly, the terms 'sum intensity' and 'mean intensity' are terms used for image in analysis in Fiji.
The definitions of "junctional" and "medial" actin were introduced by the Lecuit lab (PMID: 21068726), and we have included the appropriate reference.

4. Line 118: Supp. Fig. 2 does not have panels I and K.
5. Line 223: the authors reference data at 175 sec, but Supp. Fig. 6 does not show any images at that time point. They should be added or a different time point indicated.

These errors have been corrected.

TYPOS

1. Abstract: "[in a supracellular context" should be "in a supracellular context".

2. Line 145: should this be a reference to Supp. Fig. 5 instead of Supp. Fig. 4?
3. Line 166: I am not sure how Supp. Fig. 5 supports this statement. Is this the right figure reference? Should it be Supp. Fig. 4 instead?
4. Line 881: "representing on line" should be "representing one line".
These errors have been corrected.

OPTIONAL

Tony Harris' lab showed that the Arf-GEF Steppke antagonizes myosin and facilitates cell deformation at the leading edge of the embryonic epidermis during Drosophila dorsal closure (West et al., Curr Biol, 2017). Does Steppke localize to junctions in lateral but not central mesoderm cells? Does the pattern of Steppke localization in the mesoderm change with manipulations to the contractility of central cells?
This is certainly interesting, and we have ordered the protein trap, UAS constructs and RNAi lines. However, these will be long-term and time-consuming experiments.

Reviewer #2 (Significance (Required)):

This is an interesting study, and one that makes uses of beautiful tools, including quantitative microscopy and image analysis, mathematical modeling and optogenetic manipulations. The prediction that embryonic cells display non-linear stress-strain responses is exciting, as linearity has been the predominant assumption so far. However, I find that model predictions are not well supported by the data, and that alternative interpretations of some results are possible. Additionally, the paper lacks insight into the molecular mechanisms that facilitate stretching (although that could be the subject of a follow-up study).

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

Summary:

In this study, the authors explore potential mechanisms for why some cell constrict while other cells expand, despite similar intrinsic genetic programs, during Drosophila ventral furrow formation at the onset of gastrulation. The authors combine quantitative analyses of cell shapes and myosin levels from multiphoton confocal and Multi-View SPIM imaging, optogenetic and laser perturbation experiments, and mechanical models to argue that nonlinear mechanical interactions between cells are required to explain the cell behaviors. Based on microscopic models of the actomyosin cytoskeleton in the tissue the authors argue that the required nonlinear mechanical behavior is consistent with actomyosin network reorganization.

Major comments:

- Although the area of investigation is exciting and the results are interesting, unfortunately the quality of the results and comparison between experiment and modeling in the current version of the manuscript are not convincing. Although it is not clearly explained in the manuscript, the experimental results on cell shapes, myosin intensity, laser manipulation, optogenetic perturbations appear to be from a single

383 embryo or small number of embryos for each experiment (Figures 1, 3, 4).
384 *We had analysed a much larger number of embryos, but only included those for*
385 *presentation that provided the most extensive data. It is extremely difficult to obtain*
386 *absolutely 'perfect' embryos at high resolution for full quantification over long periods.*
387 *'Perfect' means that the embryos are mounted in such a way that they are imaged from*
388 *an angle of 45 degrees off the dorso-ventral axis, so that initially mesodermal rows 3 to*
389 *7 are seen, and then, as furrow formation progresses, the more lateral rows move*
390 *through the field of vision. It is difficult to mount in this perfect manner for two reasons:*
391 *the shape of the embryo means that the embryo does not 'like' to be balanced in this*
392 *position, but instead prefers to fall back on its side. Secondly, the embryo has to be*
393 *mounted at a time point before visible differentiation along the D-V axis, so no visual*
394 *cues exist to get the positioning right. This means that many of our recordings lack*
395 *either the more ventral or the lateral cell rows. While the findings for these more*
396 *restricted observations are fully consistent with our reports, they cannot be quantified*
397 *with a full comparison across all cell rows over the entire imaging period.*
398 *Nevertheless, we have processed and analysed further examples which we have now*
399 *included in Suppl. Fig. 2 and Suppl. Fig. 8.*
400
401 The authors state that the cell stretching pattern "was best recapitulated by a
402 superelastic response", but did not provide direct quantitative comparisons of the
403 different mechanical models to the experimental data to clearly demonstrate this.
404 *Data that illustrate this were shown in Suppl. Fig 5 – but, admittedly, were not well*
405 *explained, or rather, not at all. We have now added better explanations, expanded the*
406 *figure, included new analyses, and now present some of these data in the new Fig. 2.*
407 *Briefly, the figure shows that superelastic and elastoplastic responses are the only*
408 *curves that successfully reproduce the pattern of stretching lateral cells (last 3 cells*
409 *stretching with the inner cell stretching most and the last cell stretching least) while at*
410 *the same time matching the ratio between the cell sizes of the most stretching cells to*
411 *the least stretching cell.*
412 *The top row of the parameter scans in Suppl. Fig. 5 (now Fig. 2) shows how many cells*
413 *stretch for each combination of myosin curve steepness (y-axis) and width (x-axis) with*
414 *shades of blue indicating the number of cells, and the red outline in the field where 3*
415 *cells stretch outlining those conditions where the inner cell stretches most.*
416 *The bottom row shows the resulting size ratios of largest to smallest cell. High ratios in*
417 *the region outlined in red in the top row are only reached for the superelastic and*
418 *elastoplastic responses, with the elastomeric tending in the right direction.*
419
420 *We have now also quantified a goodness-of-fit (root mean squared error, RMSE)*
421 *measurement between our experimental data and the simulated data of all our models.*
422 *This is shown now in the new Fig. 2.*[1]
423 *We also note that only the parameter maps of the superelastic and elastoplastic*
424 *models (Fig. 2J,K) resemble the equivalent parameter maps of the microscopic model*
425 *(Fig. 3Q).*
426
427 Moreover, the local optogenetic myosin recruitment experiments in Figure 4 do not
428 provide sufficient information on optogenetic tool recruitment,
429 *We have included images that illustrate the optogenetic construct in the illuminated*
430 *cells, but not in the central cells in Suppl. Fig. 8. It is impossible to show the construct*
431 *in the 'dark' cells, because illuminating them would activate the construct.*
432

433 myosin localization,
434 *As explained above, this is unfortunately technically not feasible. The best we can do is*
435 *refer to the description of the construct by Izquierdo et al. (PMID: 29915285), which*
436 *shows the accuracy of the tool and the highly specific membrane recruitment of*
437 *myosin.*
438
439 or cell behaviors
440 *We have added quantitative comparisons between the experimental and control areas.*
441
442 to justify the claim that the central cells are not activated by the optogenetic
443 perturbation and are only responding to the forces from neighboring cells.
444
445 - The authors should provide direct quantitative comparisons of the models and
446 experiments to clearly demonstrate their claims that the superelastic model is better
447 than the linear model or other nonlinear models.
448 *See response above.*
449
450 - The authors should do additional experiments and/or provide more details for the
451 existing experiments (to include several embryos per condition) on myosin
452 quantification, photo-manipulation, and optogenetics experiments.
453 *We have provided data for more embryos for all cases.*
454
455 Additional controls would like be necessary for claims resulting from the optogenetics
456 experiments in Figure 4.
457 *This has been addressed above – we have provided additional data and controls.*
458
459 - The additional time and resources required to address these concerns would depend
460 on the experimental details, N values, and statistics in the current studies, which
461 unfortunately were not described in the current manuscript.
462 *We have been able to add substantial additional data and have added the requested*
463 *numbers. For many of the experiments each recording can be very time consuming*
464 *and for the reasons explained in this response, it is not always easy to obtain precisely*
465 *the desired recording from the desired imaging angle with the manipulations having*
466 *been done precisely in the desired position. The numbers of embryos are therefore not*
467 *high, but multiple shorter recordings provide a body of results that support the findings,*
468 *but are not easily comparable statistically.*
469
470 - Methods descriptions for reproducibility are generally adequate, with the exception of
471 N values and statistics - see above.
472 - Are the experiments adequately replicated and statistical analysis adequate? No, see
473 above.
474
475 **Minor comments:**
476
477 1) Scale bars for images are missing throughout.
478 *We have added these*
479
480 2) Number of embryos and cells analyzed missing throughout text and figure legends.
481 *We have added additional embryos for all conditions and have included the numbers of*
482 *cells analysed for all quantifications (except in cases where each data point represents*

483 *a cell).*
484
485 3) Units are missing for many quantities in figures and tables throughout.
486 *We have added these*
487
488 4) Many figure references in the main text are incorrect, pointing either to the wrong
489 figure or wrong figure panel.
490 *These have been corrected*
491
492 5) Line 728. What time point was used for myosin concentrations used in the model?
493 *We have added this information to the figure legend.*
494
495 How might myosin dynamics influence these findings?
496 *As regards the subcellular dynamics of myosin, these are included in the microscopic*
497 *model (see ref Belmonte et al.;PMID: 28954810). Preliminary results showed that small*
498 *changes in myosin stall force and unloaded myosin speed have little effect in our*
499 *general results. This is now shown in a new supplemental figure (Suppl. Fig. 6).*
500 *However, if the referee is referring to the dynamics of myosin accumulation over time,*
501 *this is an interesting question.*
502 *We had begun to explore this topic, but then realized for the linear stress-strain model*
503 *that it is in fact expected that myosin accumulation would ultimately <u>not</u> affect the*
504 *outcome. This is because in a linear model the final state of the system is determined*
505 *by the final shape of the governing myosin profile regardless of the time evolution of*
506 *the profile, and our simulations confirm this. A systematic analysis for all other stress-*
507 *strain curves with temporal changes in myosin profiles (where a dependency on the*
508 *profile temporal evolution is expected) is very time-consuming and will be interesting to*
509 *pursue in future.*
510 *The main conclusion here that linear models do not recapitulate the observed data as*
511 *well as the non-linear ones stands regardless of how the temporal dynamics of myosin*
512 *accumulation may affect the non-linear systems.*
513
514 6) The authors show a few examples of myosin pulsing in lateral cells and then
515 conclude that myosin pulsing is not qualitatively different from central cells (lines 135-
516 136). The author should quantify the number of pulsing lateral cells as well as period
517 and amplitude of pulsing, or discuss relevant results from prior studies in more detail to
518 justify this conclusion.
519 *By ‘not qualitatively different’ we had meant only ‘in the sense that they are capable of*
520 *generating contractile forces’, and we have made that more explicit in the text now. The*
521 *quantitative differences have already been analysed and reported by the Martin lab*
522 *(https://doi.org/10.1101/2020.04.15.043893; the pulses are slower and less persistent),*
523 *and our point was that in spite of these known differences, the pulses are able to*
524 *mediate constriction.*
525
526 7) Lines 145-150. The authors very briefly describe the results of the linear-stress
527 strain response and conclude this did not yield outputs corresponding to in vivo data
528 and leave this largely to the supplementary figures. This is a key point in the paper and
529 deserves much more discussion and space in the main text.
530 *We have included a more extensive description and interpretation of the results in the*
531 *main text, as detailed in several responses above*
532

533 As mentioned in main comments above, a quantitative comparison of the different
534 mechanical models to show that the superelastic model better describes the
535 observations should be included (potentially as an inset to Fig 2D showing a
536 quantitative measure of the quality of model fit to the data).
537 *These comparisons have now been expanded and explained more extensively and*
538 *moved to the main Figures.*
539
540 8) Lines 162-163. Provide more rationale for why strain-softening would most likely
541 manifest as permanent or reversible cytoskeletal reorganization.
542 *The only component of the cell that can likely mediate this physical property and also*
543 *respond at the observed time scales is the cytoskeleton. In these cells it is the main*
544 *mechanical determinant. Other components that could in principle contribute to the*
545 *nonlinearity of stress-strain response might be the viscosity of the cytosol, or the*
546 *plasma membrane. However, stress responses of fluids to shear are usually in the*
547 *direction of increasing stiffness, and rarely, if ever, with shear thinning. The same is*
548 *mostly true for colloidal solutions. Therefore it is more likely that the stress-strain*
549 *relationships at the apical surface of the cells are dominated by the dynamics of the*
550 *actin cytoskeleton given that even the shape of the plasma membrane is in general*
551 *determined by the cytoskeleton.*
552 *We have added a note to this effect in the text.*
553
554 9) Lines 187-188. "This shows that forces acting on each cell from its neighbors have
555 an important role in determining the cell's behavior." This seems somewhat obvious;
556 perhaps a bit more explanation would help the reader to understand the importance of
557 these results.
558 *We have expanded the explanations of these findings and added a sentence to relate*
559 *them to the main model of the paper*
560
561 10) Lines 196-198. How were the concentrations and lengths of F-actin chosen? How
562 were the concentration and properties of linkers chosen?
563 *The parameters were chosen on the basis of our earlier studies on simulated*
564 *contractile meshworks and the theory underlying their behaviour. We had reported the*
565 *conditions under which such meshes are able to contract, and also shown that the*
566 *underlying theory correctly predicts behaviour of experimental meshworks (for those*
567 *few conditions for which they have been reported).*
568 *Unfortunately, there are practically no measurements for the length of F-actin filaments*
569 *in vivo and estimates vary widely. Reliable data on the density of the cortical network*
570 *are equally sparse.*
571 *Based on our own previous work we chose concentrations of cross-linkers, myosin*
572 *motors and transmembrane connectors that are able to ensure optimal contraction and*
573 *force. Our in vivo measurements reported here show that the amounts of F-actin do not*
574 *vary significantly across the mesoderm, so we used the same concentration of actin,*
575 *crosslinkers and membrane connectors in all cells of the model, varying only myosin*
576 *concentration. Taking into account the cell diameter of the mesodermal cells (~7um)*
577 *and to ensure that the meshwork is sufficiently cross-connected (dense) to generate*
578 *contraction and transmit forces between cells we used a model where each cell*
579 *contains 800 F-actin filaments of 1.5 um.*
580 *We have expanded our supplemental material to make these points clearer.*
581
582 How sensitive are the results to these details of the cytoskeletal composition?

583 *We varied both the amounts of cytoskeletal components and the parameters controlling*
584 *their dynamics (such as myosin stall force and viscosity) and found little impact on*
585 *model predictions. These data are now presented in Suppl Fig. 6.*
586
587 11) Lines 238-244. It would be helpful to include some additional quantification that
588 clearly shows the reader the differences in cell behaviors in control and perturbed
589 tissue.
590 *We have added quantitative comparisons of the cells in the perturbed region with cells*
591 *in an equivalent control region, together with evaluations of two additional embryos.*
592
593 For the optogenetics experiment, it would be important to show quantification that the
594 lateral cells are not being directly perturbed during photoactivation of neighboring cells
595 (e.g. due to light leakage).
596 *We have included this information, as described above.*
597
598 In both perturbations, it would be helpful to quantify how many cells in rows 7 and 8
599 constricted and by how much did they constrict? How reproducible were these effects?
600 *The perturbation experiments were those where it was most difficult to obtain a large*
601 *number of identical-looking embryos that would allow broad statistics to be applied. For*
602 *this to work, we would have to have embryos that were identically mounted and*
603 *illuminated in the identical area of precisely rows 1 to 6 on each side of the midline – at*
604 *a resolution of one cell row of 6.2 um width. And all this blind, because at the start of*
605 *the manipulation there are no visual cues for orientation. Morphology gives no cues at*
606 *this stage. The MS2-MCP-GFP works for laser ablations, but cannot be used for the*
607 *optogenetics, because the embryo must not be exposed to blue light. This means we*
608 *cannot predetermine precisely which rows we target.*
609 *We have however added data and quantifications for the control and two further laser-*
610 *manipulated embryos, which are now shown in suppl. Fig. 8. It is evident from both that*
611 *our perturbations were slightly asymmetric and included the outer rows on only one*
612 *side and on that side several cells that would normally have stretched are now strongly*
613 *constricted. While by no means true for all lateral cells, this is a case of one black swan*
614 *disproving the hypothesis that all swans are white: any constricting cell within two cell*
615 *diameters of the mesectoderm, i.e. ones that would normally stretch proves that lateral*
616 *cells do have the capacity to constrict.*
617
618 12) Lines 245-252. A key assumption in interpreting this experiment seems to be that
619 the central cells are not directly perturbed by the optogenetic activation. Additional
620 quantifications of RhoGEF2-CRY2 and/or myosin should be shown to support this.
621 *We have included an image of the optogenetically activated construct in this*
622 *experiment in Fig. 5, but we cannot show its behaviour in the non-activated part*
623 *because if we illuminated it, it would be activated. We were unable to create the*
624 *embryos necessary to document the behaviour of myosin.*
625 It would be helpful to include some additional quantification that clearly shows the
626 reader the differences in cell behaviors in control and experimental regions. How
627 reproducible were these effects?
628 *We now provide the results from two additional embryos in Suppl. Fig. 8, and include*
629 *quantitative comparisons between the control and experimental regions for these and*
630 *for the embryos that are currently shown in Fig. 5 E.*
631
632 13) A section on statistics is missing from the methods section.

633 *We have added descriptions of the quantifications and statistics.*
634
635 14) Line 615. Ensure that Eq. 1 is dimensionally consistent; crucially, what units are
636 used for 'M'? If the model is non-dimensionalized, provide the reference scales.
637 *Apart from the initial distance between membrane positions (set to 6.2 um) all other*
638 *units in our visco-elastic model are arbitrary. In order to make this clearer, instead of*
639 *using the term "viscosity" in equation 1, we now call it a "damping constant".*
640
641 15) Line 675: The investigated stress-strain relationships are presented in Table S1.
642 What are the definitions of xpl and xsh?
643 *We have included these definitions in materials and methods:*
644 *All stress-strain curves are linear for extensive strains (Δx) lower than the*
645 *proportionality limit (x_{pl}), with some curves (elastoplastic and superelastic) undergoing*
646 *a strain-softening to strain-hardening change after a given strain-hardening limit (x_{sh}).*
647
648 16) Line 678: Parameter values for the stress-strain relationships are given in Table
649 S2. Can you provide more information on how these values were selected and their
650 units? How sensitive are the results to changes in these values? Provide references
651 when possible.
652 *The values for xpl and xsh were chosen to be within the range of the observed lengths*
653 *of stretching cells, with xpl < xsh. Changing the values of each parameter listed in*
654 *Table S2 does change the results quantitatively, but over the ranges we tested them,*
655 *never to the point of making the linear or the other non-linear models reproduce the*
656 *target pattern of stretching.*
657 *We have stated this in the materials and methods section.*
658
659 17) Line 697. Please comment on why the embryo appears skewed to the right.
660 *Embryos are not always 'perfect', unfortunately. In addition, they can get slightly*
661 *squashed during mounting and imaging. In spite of its imperfection, we showed this*
662 *particular one, because we had imaging data for a long period without drift or other*
663 *interference, and with good contrast at great depth.*
664
665 18) Line 712. A color-bar corresponding to this color-code is missing in the figure.
666 *This has been corrected.*
667
668 19) Lines 715-717. It seems panels E and E' are swapped in the legend.
669 *corrected*
670
671 20) Line 724 (Fig 2). It is difficult to read anything in panel K inset or Panel L inset.
672 *We have rearranged this figure and replaced some panels for greater clarity, and to*
673 *remove redundancy.*
674
675 21) Line 728. What does "embryo 1" refer to?
676 *This was a remainder from an old plan where each embryo was numbered and listed in*
677 *a table so that it could be cross-referred to. We have now described in the*
678 *supplementary table the genotypes and imaging technique for each group of embryos.*
679 *Where we show data or analyses of the same embryo in different figures, we refer*
680 *directly to the relevant panels. We have made sure the embryos are referred to*
681 *correctly in the figure legends.*
682

22) Line 732. A quantitative measure of the quality of the fits of the models to the experimental data should be included.
We have done this, and the new data are now included in the new Figure 2.

23) Line 739. What exactly does "Embryo 2" refer to?
See comment 21

24) Line 779. Why is a z-plane of 15 microns below surface chosen?
25) Line 797. Why is a z-plane of 25 microns below the surface chosen?
The planes were chosen in each case to show the reader in one single plane rows 7 and 8 along with the central cells

26) Line 900. Panel G in Supp Fig 5 is not described in figure description.
The panel captions were wrongly numbered. This has now been corrected, and more information on this figure has been included in the text.

- Are prior studies referenced appropriately? Yes.
- Are the text and figures clear and accurate? No (see details listed above).
- It would be very helpful to the reader to show direct quantitative comparison of the different mechanical models with the experimental observations to show how much better the nonlinear model is compared to the linear model.

We have included this.

An extended explanation of experiments and experimental results within the main text would improve the manuscript.
We have expanded our explanations in many places.

Reviewer #3 (Significance (Required)):

The key advance in this work is in identifying a potential role of nonlinear mechanical properties in contributing to distinct cell behaviors within a tissue during development in vivo. This contributes to a growing body of work highlighting the importance of cell and tissue mechanical properties in regulating cell behaviors during the formation of tissue structure.

This work adds to a growing body of work connecting actomyosin contractility in cells to tissue-scale behavior during development. This work provides a unique mechanical modeling perspective to the study of apical constriction during Drosophila ventral furrow invagination, highlighting a potential role for superelastic cell mechanical behaviors during morphogenesis in vivo.

The finding would be of interest to researchers working in the areas of morphogenesis, mechanobiology, the cytoskeleton, and active matter.

This reviewer's expertise is in experimental studies of the cytoskeleton and cell mechanics during morphogenesis.

June 21, 2021

RE: JCB Manuscript #202104107T

Prof. Maria Leptin
European Molecular Biology Organization
Meyerhofstraße 1
Heidelberg 69117
Germany

Dear Maria -

We have now received feedback from two external reviewers on your revised manuscript "Mechanical competition alters the cellular interpretation of an endogenous genetic programme". I am pleased to be able to report that they each feel the study is significantly improved and addresses many of the major and minor issues that were raised in the initial reviews. However, they raise several points that you will need to respond to before the manuscript can be accepted by JCB. Reviewer #1 is still concerned about the small number of embryos in some of the experiments, while acknowledging that they are very challenging to perform. They suggest that you include some discussion in the main text about these experimental challenges and comment on the small value of n and its impact on the conclusions. This reviewer also asks for more detail on the optogenetic experiments. Reviewer #2 noted that the revised version addresses most of their comments but still has a concern about some of the citations. For instance, several of the citations about the use of an infrared laser did not demonstrate viability or cell integrity, and one uses a uv laser rather than an ir laser.

Overall, while we cannot accept the manuscript in its present form, we feel that the remaining issues can be addressed largely by changes to the text. We look forward to receiving a suitably revised version. Please note that we will need a point-by-point response to the comments of the reviewers.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles and Tools may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset

magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

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

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the

findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Sincerely,

Ian Macara, Ph.D.
Editor

Andrea L. Marat, Ph.D.
Senior Scientific Editor

Journal of Cell Biology

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

In this study, the authors explore potential mechanisms for why some cell constrict while other cells expand, despite similar intrinsic genetic programs, during Drosophila ventral furrow formation at the onset of gastrulation. The authors combine quantitative analyses of cell shapes and myosin levels from multiphoton confocal and Multi-View SPIM imaging, optogenetic and laser perturbation experiments, and mechanical models to argue that nonlinear mechanical interactions between cells are required to explain the cell behaviors. Based on microscopic models of the actomyosin cytoskeleton in the tissue the authors argue that the required nonlinear mechanical behavior is consistent with actomyosin network reorganization.

The current revision addresses many of the major and minor issues raised by the reviewers. This has strengthened support for the main claim of the paper and improved the readability of the manuscript. However, several issues remain that should be addressed.

MAJOR:

- Although the authors have importantly expanded the set of embryos studied, including additional data from embryos in the supplement (Suppl Fig. 2 and Suppl Fig. 8), some crucial results in the main text still appear to represent data from only one embryo (e.g. Figures 1F-H; 2A,E,F; 3G-K; 4; 5). Many of these experiments are very challenging, as the authors explain in their response, but why not try to pull together the data from these additional embryos and provide some statistics in the main manuscript? If this is not possible, the authors should include some discussion in the main text of these experimental challenges and comment on the fact that some results are based on a small number of experiments, potentially weakening some of the conclusions.

- The experimental details of the ablation and optogenetics experiments in Fig. 4 and Fig. 5 are not clearly explained in the current revision. For example, for the optogenetic manipulations: What was the duration of the light activation? Was it a single activation or continuous activation throughout the experiment? What is the spatial resolution of activation (e.g. single cell, sub-cellular, etc)? It is difficult to assess these results without this information.

- Further analysis and explanation of the ablation and optogenetics experiments in Fig. 4 are needed. It is difficult for the reader to directly and quantitatively compare the differences in cell areas between Fig 4B', D', and K', making it challenging to assess their interpretation of these

results in the main text. The authors should provide more explanation of the OCRL optogenetic tools and its potential effects on F-actin and cell adhesion; it seems overly simplistic to say it "inactivates the actomyosin network".

- Further discussion of the optogenetics experiments in Fig. 5 is needed. Importantly, the authors cannot do experiments to visualize activation of the tool or the effects on myosin and only have a small number of embryos. These facts, as well as other potential interpretations of these data, should be discussed in the main text to help the reader evaluate conclusions based on this data.

- In the response to reviewers, the authors provide lots of helpful discussion of results, but have not incorporated much of this into the manuscript. The manuscript would be improved by a much more detailed and nuanced Discussion section.

MINOR

- Fig 3: Labels missing from colorbars in I and J.
- Fig 4: Color codes missing from many panels.
- Fig 4H,I: Difficult to assess localization patterns with overlaid yellow regions.
- Fig 5: Cell area color codes missing.
- Supp Fig 2: Very difficult to read most text in F-H
- Supp Fig 8: Very difficult to read most text in B

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

The authors have attempted to address most of my comments. My main remaining concern is with the quality and appropriateness of some of the citations. These should be fixed prior to publication:

1. In their introduction, the authors argue that differences in gene expression cannot explain differences in cell behaviour (constrict or stretch) during mesoderm invagination in Drosophila. Further, in the results section (page 5), they discuss how junctional actin changes occur before cell shape changes in the mesoderm begin, and apical actin is present in both central and lateral mesoderm cells during furrow formation. Later on, the authors cite a preprint from Adam Martin's lab (ref. 47) that has now been published in Development. That study suggests that quantitative differences in gene expression do lead to a pattern of F-actin distribution associated with whether cells constrict or stretch their apex. These results and their similarities and differences with those in the current study should be acknowledged and discussed.

2. Line 274: "[...] with a pulsed infrared laser, a method that does not compromise the cells' integrity or viability13,16,38.". I would remove this sentence.The authors argue here and in their response to this reviewer that the cells are not wounded by the infrared laser, but they have no evidence to support that. The authors never show that the cell integrity or viability are intact. Other groups have used laser ablation before to prevent the constriction of a cell, and claiming cell integrity or viability is unnecessary (and possibly wrong). Furthermore, the references selected do not support the claim. In reference 13 the authors used an ultraviolet laser, not an infrared one, and they never tested for membrane integrity or cell viability; and in reference 38 they never used laser ablation, but magnetic tweezers. In reference 16 the authors used an infrared laser, but I could not find any evidence there to demonstrate that cell integrity or viability were not compromised. The Lecuit lab showed that 3-photon irradiation with an infrared laser preserves membrane integrity (Cavey et al.,

2008), but again, cell viability was not assessed.

3. Lines 214-216. Ulrich Tepass has previously shown that the forces acting on a cell from its neighbours determine the rate of apical constriction (Simoes et al., J Cell Biol, 2017). This paper should be cited.

4. In their letter, the authors argue that "The definitions of "junctional" and "medial" actin were introduced by the Lecuit lab (PMID: 21068726), and we have included the appropriate reference." However, it was Eric Wieschaus who first used those terms and should be referenced (https://www.nature.com/articles/nature07522).

5. This is completely optional, but in my opinion, the discussion of non-linear stress-strain responses in page 6 would benefit from a figure displaying the stress-strain graphs for the four responses tested. Otherwise, expressions like "with a decrease in stiffness after the proportional limit, but no strain-softening" are unclear (isn't a decrease in stiffness a softening?).

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In this study, the authors explore potential mechanisms for why some cell constrict while other cells expand, despite similar intrinsic genetic programs, during Drosophila ventral furrow formation at the onset of gastrulation. The authors combine quantitative analyses of cell shapes and myosin levels from multiphoton confocal and Multi-View SPIM imaging, optogenetic and laser perturbation experiments, and mechanical models to argue that nonlinear mechanical interactions between cells are required to explain the cell behaviors. Based on microscopic models of the actomyosin cytoskeleton in the tissue the authors argue that the required nonlinear mechanical behavior is consistent with actomyosin network reorganization.

The current revision addresses many of the major and minor issues raised by the reviewers. This has strengthened support for the main claim of the paper and improved the readability of the manuscript. However, several issues remain that should be addressed.

MAJOR:

- Although the authors have importantly expanded the set of embryos studied, including additional data from embryos in the supplement (Suppl Fig. 2 and Suppl Fig. 8), some crucial results in the main text still appear to represent data from only one embryo (e.g. Figures 1F-H; 2A,E,F; 3G-K; 4; 5). Many of these experiments are very challenging, as the authors explain in their response, but why not try to pull together the data from these additional embryos and provide some statistics in the main manuscript?

Because each embryo is slightly different from the next, we feel that combining them all and doing a statistic comparison may not be justified. This is particularly true for the laser and optogenetic perturbations, where the two sides of the embryo can be different. Choosing only the 'affected' side could be construed as misrepresentation (while averaging data across the unaffected sides as well would not make sense), and we therefore prefer to show each individual experiment, so the readers can get their own impression and make their own judgement. We have therefore taken the route suggested below by this referee and added more explanation in the text. We have however also included a figure containing the data of all embryos corresponding to those in Figs. 1 and 3, and have provided a table with the results of all experimental perturbations to make them easier to compare, as requested below.

If this is not possible, the authors should include some discussion in the main text of these experimental challenges and comment on the fact that some results are based on a small number of experiments, potentially weakening some of the conclusions.

We have done this as well.

- The experimental details of the ablation and optogenetics experiments in Fig. 4 and Fig. 5 are not clearly explained in the current revision. For example, for the optogenetic manipulations: What was the duration of the light activation? Was it a single activation or continuous activation throughout the experiment? What is the spatial resolution of activation (e.g. single cell, sub-cellular, etc)? It is difficult to assess these results without this information.

We have added this information in the materials and methods section.

- Further analysis and explanation of the ablation and optogenetics experiments in Fig. 4 are needed. It is difficult for the reader to directly and quantitatively compare the differences in cell areas between Fig 4B', D', and K', making it challenging to assess their interpretation of these results in the main text.

See above – we have now combined all these data in a table.

The authors should provide more explanation of the OCRL optogenetic tools and its potential effects on F-actin and cell adhesion; it seems overly simplistic to say it "inactivates the actomyosin network".

We have done this.

- Further discussion of the optogenetics experiments in Fig. 5 is needed. Importantly, the authors cannot do experiments to visualize activation of the tool or the effects on myosin and only have a small number of embryos. These facts, as well as other potential interpretations of these data, should be discussed in the main text to help the reader evaluate conclusions based on this data.

- In the response to reviewers, the authors provide lots of helpful discussion of results, but have not incorporated much of this into the manuscript. The manuscript would be improved by a much more detailed and nuanced Discussion section.

We have added further explanations and mentioned constraints and caveats.
We also note that because the referee reports as well as our responses are publicly posted both on biorXiv and ultimately on the journal site, the reader will have access to the entirety of these useful exchanges.

MINOR

- Fig 3: Labels missing from colorbars in I and J.
- Fig 4: Color codes missing from many panels.
- Fig 4H,I: Difficult to assess localization patterns with overlaid yellow regions.
- Fig 5: Cell area color codes missing.
- Supp Fig 2: Very difficult to read most text in F-H
- Supp Fig 8: Very difficult to read most text in B

All done.

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

The authors have attempted to address most of my comments. My main remaining concern is with the quality and appropriateness of some of the citations. These should be fixed prior to publication:

1. In their introduction, the authors argue that differences in gene expression cannot explain differences in cell behaviour (constrict or stretch) during mesoderm invagination in Drosophila. Further, in the results section (page 5), they discuss how junctional actin changes occur before cell shape changes in the mesoderm begin, and apical actin is present in both central and lateral mesoderm cells during furrow formation. Later on, the authors cite a preprint from Adam Martin's lab (ref. 47) that has now been published in Development. That study suggests that quantitative differences in gene expression do lead to a pattern of F-actin distribution associated with whether cells constrict or stretch their

apex. These results and their similarities and differences with those in the current study should be acknowledged and discussed.

These results beautifully complement ours and we have now included them in the discussion.

2. Line 274: "[...] with a pulsed infrared laser, a method that does not compromise the cells' integrity or viability13,16,38.". I would remove this sentence.The authors argue here and in their response to this reviewer that the cells are not wounded by the infrared laser, but they have no evidence to support that. The authors never show that the cell integrity or viability are intact. Other groups have used laser ablation before to prevent the constriction of a cell, and claiming cell integrity or viability is unnecessary (and possibly wrong). Furthermore, the references selected do not support the claim. In reference 13 the authors used an ultraviolet laser, not an infrared one, and they never tested for membrane integrity or cell viability; and in reference 38 they never used laser ablation, but magnetic tweezers. In reference 16 the authors used an infrared laser, but I could not find any evidence there to demonstrate that cell integrity or viability were not compromised. The Lecuit lab showed that 3-photon irradiation with an infrared laser preserves membrane integrity (Cavey et al., 2008), but again, cell viability was not assessed.

We regret the inaccurate citations, and we have followed the referee's suggestion to delete this clause.
With regard to cell viability, we have now added the following note in the materials section: "The laser treatment does not kill cells or permanently damage the cytoskeleton as shown by the fact that they re-constrict and continue to participate in furrow formation once the illumination stops".

3. Lines 214-216. Ulrich Tepass has previously shown that the forces acting on a cell from its neighbours determine the rate of apical constriction (Simoes et al., J Cell Biol, 2017). This paper should be cited.

We thank the reviewer for alerting us to this superb example, which we now cite in the introduction.

4. In their letter, the authors argue that "The definitions of "junctional" and "medial" actin were introduced by the Lecuit lab (PMID: 21068726), and we have included the appropriate reference." However, it was Eric Wieschaus who first used those terms and should be referenced (https://www.nature.com/articles/nature07522).

Very good point, we have corrected this.

5. This is completely optional, but in my opinion, the discussion of non-linear stress-strain responses in page 6 would benefit from a figure displaying the stress-strain graphs for the four responses tested. Otherwise, expressions like "with a decrease in stiffness after the proportional limit, but no strain-softening" are unclear (isn't a decrease in stiffness a softening?).

We agree with the reviewer that the description may be confusing. By "strain-softening" we meant a negative slope in the stress-strain graph, corresponding to cases where the material experiences reduced stress with increasing strains. By contrast the term "decrease in stiffness" corresponds to cases where the stress strain slope is still positive, but lower (more

horizontal), corresponding to cases where the stress still increases with higher strains, but does so at reduced rates. We have added these explanations, specifically referring to the slopes shown in Fig. 2D.
For all the other responses we refer the reviewer to Figure 2D where all the curves were graphically displayed.