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Differential proliferation rates generate patterns of mechanical tension that orient tissue growth

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Editor: David del Alamo

1st Editorial Decision

03 January 2013

Thank you for the submission of your manuscript to The EMBO Journal and please accept my apologies for the delay in responding due to this recent holiday period. We have now received the full set of reports from the referees, which I copy below.

As you can see from their comments, all three referees are very positive about both the general interest and the novelty of your findings and recommend the publication of your manuscript, provided their concerns are properly addressed. In general, they are convinced that the evidence presented properly supports your conclusions, although a number of technical concerns and the need for some clarifications have arisen. Although these concerns are explicitly mentioned in the referee reports and thus I will not repeat them here, I would like to draw your attention to a few important specific points. Referee #1 believes that the relationship between circumferential tension and differential proliferation rates should be further explored, as stated in his/her report. Along the same lines, both referee #2 and #3 also think that the relationship between differential proliferation rates and cell division orientation is not sufficiently supported by your experimental evidence. In addition, how your current model compares to your previous hypothesis involving Fat and Dachsous remains unclear, in referee #2's opinion.

Taking these reports into consideration, I would like to invite you to submit a revised version of the manuscript. Please be aware that your revised manuscript must address the referees' concerns and their suggestions should be taken on board. It is 'The EMBO Journal' policy to allow a single round of revision only and, therefore, acceptance or rejection of your study will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will

form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: <http://www.nature.com/emboj/about/process.html>

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you again for the opportunity to consider your work for publication in The EMBO journal. I look forward to your revision.

Please, do not hesitate to contact me in case you have any further question, need further input or you anticipate any problem during the revision process.

REFEREE REPORTS

Referee #1

The manuscript by Mao and colleagues analyses the mechanisms by which differential proliferation rates within an epithelium can generate different patterns of mechanical tension and thereby orient tissue growth. The authors provide evidence for the existence of two distinct regions within the wing pouch - the distal region (centre) containing smaller and less elongated cells, and the most proximal regions consisting of larger and more elongated cells. In addition the authors show that in distal regions, cell division orientation and the longest cell axis are parallel to the PD axis, while in proximal regions they are oriented perpendicular to the PD axis along the circumference of the wing disc. This perpendicular/circumferential orientation in proximal regions correlates with higher circumferential tension in proximal compared to distal regions of the wing disc. Finally the authors provide both experimental and theoretical evidence that the gradient of circumferential tension along the PD axis is due to differential proliferation rates of wing disc cells along the same axis.

The manuscript is well written and contains potentially interesting observations. However, there are several points of major criticism that need to be addressed before the manuscript is suitable for publication in EMBO Journal.

1. The evidence for a PD gradient of circumferential tissue tension is interesting, but not sufficient to state there is necessarily a local tension anisotropy along the circumferential lines. In order to show tension anisotropy, the authors need to also perform cuts parallel to the circumferential lines and confirm that tissue tension perpendicular to these lines is smaller than along the circumferential lines. In addition the authors should provide stills or movies of the laser ablation experiments to judge about the actual experimental work done.
2. While the observation that there are differential proliferation rates in these two regions of the wing disc is potentially interesting, the direct influence of this observation on the tissue circumferential tension and cell topology/division orientation still remains to be elucidated *in vivo*. The authors need to demonstrate that affecting the proliferation rates (making them uniform or changing the distal/proximal ratios) *in vivo* has indeed an effect on tissue tension and consequently cell topology/cell division orientation.
3. Along the same vein, it is not clear if the observed tissue tension arises from the differential rates of division or if these rates of division only modulate pre-existing global tissue tension. For example, in case there is no external tension, one might assume that there is 'negative' tension in the center where proliferation rates are higher than in proximal/marginal regions of the disc.

Taken together, this is a potentially interesting manuscript, which needs more decisive work before it is suitable for publication in EMBO Journal.

Referee #2

Mao et al. address the question of the origin of cell shape and growth orientation in the drosophila wing disc. Their previous work, based on clone shape and cell division analysis, indicated that growth in the wing disc is oriented along a proximal-distal axis (defined as being towards central regions of the DV boundary, and that this orientation depends on the the activity of Fat and the atypical myosin Dachs. Their work, along with that of others, has shown that Dachs is intracellularly polarized towards the DV boundary and away from the hinge - at least in regions near the hinge (its polarity is less convincing in central regions of the wing pouch). They suggested previously that Dachs orients cell divisions by constricting the cell boundaries on which it is present, thereby biasing the cell division axis to coincide with the axis of cell elongation.

Here, they have looked more carefully at clone shapes and cell divisions in the wing disc, and report a slightly different finding: while growth is oriented towards the dv boundary in central regions of the wing pouch (as they previously found), it is oriented in a perpendicular direction in regions close to the hinge (i.e. growth is parallel to the hinge near the hinge). They quantify cell shapes over developmental time, and find that cells become elongated parallel to the hinge fold beginning between 48 and 72 hours. Elongation is particularly strong near the hinge (consistent with the cell division orientation in this region). They perform laser ablation studies and show that long boundaries are under more stress. They conclude that cells elongate passively due to external forces, and speculate that Dachs localizes to boundaries under tension. They then use a vertex model to explore different mechanisms that could give rise to this pattern of cell shapes and division orientations. The modeling suggests that differences in growth rate (higher in the center) might give rise to such a pattern. Indeed, when they use clone size to estimate growth rates in different regions, they find that the central wing pouch grows a bit faster at early stages. They postulate that this growth rate difference accounts for the growth pattern observed in the wing disc.

It is not clear how these findings relate to their previous model, which was that Dachs promotes growth along the PD axis by elongating cells in that direction. The authors argue in the discussion that Dachs may localize to cell boundaries in response to mechanical tension, and say that "this could allow the distal cells to continue to elongate and divide along the P-D axis whilst preventing the proximal cells from becoming excessively stretched, thus maintaining tissue integrity. The problem with this idea is that their quantifications of cell elongation in discs show that there is no region of the wing disc (also not in the middle) where cells are elongated parallel to the PD axis, so their current experiments would seem to rule out this idea. The clear prediction of their model is that cells in Fat and Dachs mutant discs would be much more highly elongated parallel to the hinge throughout the wing pouch. It would have been easy to quantify cell shapes with their current methodologies in Fat and Dachs mutant discs, but this experiment hasn't been done. It is also hard to see how Dachs could localize to cell boundaries purely as a consequence of stress, when they and others have already shown that it localizes in response to the gradient of Dachsous expression. Unless they would like to argue that Fat and Dachsous affect the differential growth rate and only indirectly polarize Dachs through effects on growth. But none of this is discussed.

The simulations also seem to indicate that cells are never elongated in the "PD" axis, at least if I understand Figure 6F correctly. While the cell elongation ratio does decrease from the outside to the center of the field of cells, it is still greater than 1 in the center (I am assuming they are always measuring the elongation perpendicular to the PD axis - I can't find this information anywhere and if not, it should be explicitly stated). An assumption in their vertex model is that cells would prefer to divide along their long axis, so how do clone shapes end up being parallel to the PD axis? Are there T1 transitions that are predicted by the model? If so, have the authors observed them in vivo? These things need to be more carefully explained.

While the image analysis and modeling are interesting, the authors do not do enough to test the functional importance of differential growth rates for growth orientation, and do not satisfyingly relate their current observations to their previous model accounting for the effects of Dachs on growth orientation. I would therefore not support publication.

Specific questions/points:

- While the authors present a very nice analysis of cell geometry, tissue organization and regional differences in proliferation rates over time, other analyses are not as carefully done with respect to

time. Thus, it is not completely explained how the dynamics of growth actually work. Specifically:

- Live imaging: quantitation of cell division orientations was only done in the late discs (not actually specified, but since they mention hinge folds, it must be late).
- Boundary tension measurements: what stage of development was used for the laser ablation experiments? This is not mentioned, but seems vital to the argument that global forces are inducing anisotropies in cell elongation. Even better would be to have laser ablations over time to see when this difference arises and how that correlates to the evolution of anisotropies in cell area and polarization of phospho MyoII.
- Dachs polarization: I guess it is assumed to be present and polarized throughout growth, but previous papers have only looked at relatively late stages (?). When is Dachs polarization actually evident? Does it change over time? How does that relate to the transitions observed in tissue/cell geometry?
- In general, it needs to be explicitly written which experiments were live vs fixed and what stages were used for the analysis.
- The paper would be much stronger with an analysis of cell shape and growth in the absence of Dachs (I.e, RNAi or mutant). Also, the simulations seem to already have Dachs built into them as an assumption - what happens if they take it out - how does it compare to Fat or Dachs mutants?
- While the quantitative approach is admirable, the presentation of the data needs to be more consistent to help the reader to make comparisons. The reader is currently forced to compare line graphs with box+whisker or heat maps of cells in a 2D representation (and yet additional plotting methods in the Supplement). Not ideal and very subjective. See specific notes in the Figures section below.

- In Figure 2, I gather from the figure legend that these were fixed samples (since it is stated that the nubbin expression domain was used for selecting the analysis region). I'm not sure why they would use immunofluorescence when they have the ability to culture discs *ex vivo* and image live to watch how elongation and division angles are coordinated, as well as length of cell cycle.

- Modeling:

- p7/Fig 5: why not also try introducing PD division axis bias in your model to see how this influences tissue/cell geometry?
- p7: When does the hinge fold develop (not obvious to those not studying *Drosophila* wing development)? From the images, best guess is between 72-96hr. AEL If the hinge fold were to be the origin of global forces acting on the whole pouch, it would have to be present before 72hr AEL, when the first anisotropies in area/elongation were noted. This point should be mentioned.
- Were T1 transitions allowed in the model? Presumably yes, since they were in the last paper. Were they visualized in the live imaging? How many T1 transitions occur in the different scenarios (if any), and what happens if you don't allow rearrangements. Should be discussed.
- What happens in the different scenarios when Dachs is not included in the line tension?
- Division orientation in simulated scenario that's most relevant to the *in vivo* scenario does not really seem to match experimental results. There is a much shallower difference in orientation of cell divisions along PD axis in the simulations compared with *in vivo*, even though the clone orientation agrees better (compare Fig 6J with 1F). This seems to contradict their model and suggest that additional elements are involved. It also suggests that T1 transitions contribute significantly to clone orientation in the simulations -is this correct? Again, does this occur *in vivo*?

Minor points:

- p2: "Morphogenesis then further sculpts this post-mitotic tissue mass ...". The first two references following this statement clearly show that a tissue is NOT (necessarily) post-mitotic during morphogenesis (in fact the divisions are likely highly important for tissue shear). This is a classic error of confusing growth (increase in mass) with division (splitting of two cells), which can be separable.
- p5: "At 48 hrs, the cells in the wing pouch are largely uniform in cell area and elongation...". Actually in the 48 hr timepoint, the error bars are by far the largest of all your timepoints, indicated a larger variability (not uniform) in cell area. You could say instead that there is less of a P-D bias in cell area, but don't say that they are "largely uniform in cell area". Indeed the drop in variation is probably meaningful, although SEM is not the correct measurement of variation in that case. What does the standard deviation look like?
- p5-6/Fig 3: The pattern of orientation actually does seem to change slightly between 96-120 around the center of the pouch.
- p6: "This cell-autonomous anisotropy of Myo-II localization would be expected..."? How do you

know it's cell-autonomous? Maybe the wording is confusing? Better to say "If the anisotropy in MyoII activity was cell-autonomous, it would be expected to ..."

- p8: "Hence, the constriction ring is providing a physical force that does affect cell size ..." This is slightly misleading. You can say it is one possible solution, but this statement implies that this is the mechanism *in vivo* as well, which was not demonstrated.
- Should probably address in the Discussion the biochemical-based models that have been proposed to explain how Dpp regulates even proliferation in the disc.

Figures:

Fig 1:

- It would be more useful to make B+C correspond to the example in A. Both in shape of overall pouch and in clones.
- Why are there no clones in the center?
- In the particular examples of B+C, it appears that the elongation of the clones is less pronounced than in the distal center. Is that generally true? In the measurements of clone orientation, is there any normalization for amount of clone elongation?
- Likewise for cell elongation. Since cells in the center are less elongated, isn't it somewhat irrelevant for these cells? Showing orientation without extent of elongation is not as useful.
- Why not also absolute distance from the center? Not clear which is more important for the mechanics.

Fig 2:

- Why don't you show nubbin expression, since this was (according to the legend) used to define the region of analysis (at least in young discs)?

Fig 3:

- In part C, the legend says that the length of the bar indicates the extent of elongation, but all the bars look nearly the same length. Certainly the differences are far less obvious than the color coding in part B. Either add color or change the scale to include a higher dynamic range.
- Also part C, it would be really useful if you could illustrate somehow (maybe with colored rings) where 0.1, 0.3, 0.5, 0.7, 0.9 "relative distances" are on this diagram so that it could more easily be compared to the plots of division orientation elsewhere in the paper. OR, even better, you could also plot cell elongation ratio and orientations in a way that makes it easier to compare to division orientation (I.e, also in a box+whisker plot as a function of distance from center).
- In D+E, the error bars seem a bit too small. What does the standard deviation look like?
- Why are the data in D+E presented in line graphs? Better would be a scatter plot or box+whisker as the other data.

Fig 5:

- Why is cell area so much more variable in situation 2 and 3 (looking at the images in part A)? (Again, this may be a reason to look at standard deviation rather than SEM).

Fig 6:

- Also show areas and elongation ratio for last simulated scenario (72hr as in I-K)
- Part K: really difficult to see the patterns of clones. Can you make a diagram that looks more like the experiment, where not all cells are labeled (just some clones, rest white)? Can you quantify clone shape and size (as you did in last paper perhaps) and compare to experiment with respect to orientation along PD axis? That would make it easier to compare *in vivo* with *in silico* experiments.
- For E+F see note above for Fig 3.

Fig S2 is unnecessary

Fig S3 should probably be in the main section of the paper.

Methods:

- p14: in "fixed sample imaging" the 0.5-1mm intervals are probably meant to be in microns not mm.
- p14: what stage of discs were imaged and used to quantitate division angles?
- What stage discs were used for the laser ablation experiments?

Referee #3

In the manuscript "Differential proliferation rates generate patterns of mechanical tension that orient tissue growth" by Mao et al., the authors carefully describe cell division dynamics in the proliferating *Drosophila* imaginal disc. Based on differential rates of proliferation in proximal and distal aspects of the wing pouch, the authors argue that a global tension pattern emerges, and that this tension pattern influences cell division orientation. This work provides a new perspective on the interaction between individual cells and tissue-level forces in the determination of tissue architecture. Overall, it is crisply written and should be of some interest to a broad audience.

I have several major concerns, however, about the manuscript in its present form. These concerns should be addressed by modifications to the text and new experiments.

1. In the first results section on page 4, it is a bit unclear exactly which part of the presumptive wing is being analyzed. If it is only the central region, it would be helpful to know what fraction of the wing blade is actually generated from this central domain. Further, part of the idea here is that the deep tissue clefts surrounding the wing blade primordium constitute a physical barrier. How do rates of proliferation and orientations of division look within the folds? Although somewhat complex to analyze, it should be possible to compare the behavior of cells in the folds with those of the blade region. Are they subjected to a different set of forces as well? What fraction of the presumptive wing blade is actually generated from cells in the folds?

2. In the second results section beginning on page 5, the authors begin to use the term "epithelial topology" when they should be using the more appropriate term "epithelial geometry." Topology singularly refers to the global system of cell-neighbor relationships without taking into consideration apical surface areas, side lengths, elongation, cell volumes, etc. This could cause some confusion and should be revised throughout the paper.

3. On page 6, the authors should more clearly state the four distinct mechanisms since the way it's written initially makes it sound like two mechanisms.

In the analysis of Ecad, Sqh, and p-Sqh in Fig S3, it would be helpful to see the localization relative to a uniform cortical or plasma membrane marker in addition to the individual channels.

Could the authors explain more clearly how an external force might lead to localized p-Myo II localization?

4. Without commenting on the modeling itself, the authors use *in silico* methods to validate their general hypothesis- which is that spatially differential growth rates lead to corresponding spatial patterns of cell elongation and division orientation in the wing disc. In order to more directly explore this idea, the authors should generate local changes in proliferation rate in order to experimentally validate any corresponding local effects on cell division orientation. This could be done either by locally perturbing rates of cell proliferation directly, or by altering spatial patterning cues that set up the p-d axis and then examining the effects on cell division orientation.

Referee #1

1. The evidence for a PD gradient of circumferential tissue tension is interesting, but not sufficient to state there is necessarily a local tension anisotropy along the circumferential lines. In order to show tension anisotropy, the authors need to also perform cuts parallel to the circumferential lines and confirm that tissue tension perpendicular to these lines is smaller than along the circumferential lines.

As requested, we have performed laser ablations of lateral junctions (perpendicular to the P-D junctions). These experiments show that, in proximal cells, the P/D junctions are clearly under more tension than the lateral junction (Fig. 4B-G – initial vertex recoil velocity ratio (P/D-lateral) = 1.87). This difference is less pronounced in distal cells (initial vertex recoil velocity ratio (P/D-lateral) = 1.4). These data confirm our conclusion that proximal cells are under more anisotropic tension. See also text p6 for details.

2. In addition the authors should provide stills or movies of the laser ablation experiments to judge about the actual experimental work done.

We now provide stills from a typical laser ablation (Fig. 4F-F'') as well as a time-lapse movie (Supplementary movie S2).

3. While the observation that there are differential proliferation rates in these two regions of the wing disc is potentially interesting, the direct influence of this observation on the tissue circumferential tension and cell topology/division orientation still remains to be elucidated in vivo. The authors need to demonstrate that affecting the proliferation rates (making them uniform or changing the distal/proximal ratios) in vivo has indeed an effect on tissue tension and consequently cell topology/cell division orientation.

Although ideally we would be able to completely flatten the endogenous proliferation gradient and show the cells are no longer stretched at the periphery, in practice, this is impossible to achieve. Firstly, we would need to find a *GAL4* driver with the exact spatial and temporal pattern needed to equilibrate proliferation rates at the time when proliferation is graded. Secondly we would need to express a growth regulator or growth inhibitor at precisely the right level in the right pattern of cells to obtain a completely even proliferation rate. We have tried a number of drivers, but none had the desired characteristics (*nub-GAL4*, *tsh-GAL4*, *rn-GAL4*, *vg^{BE}-GAL4*, *hth-GAL4*...). For example wing pouch drivers such as *rn-GAL4* or *nub-GAL4* are expressed too late to affect proliferation on time to prevent the differential from arising. We tried several combinations, but unfortunately none allowed us to observe the desired flattening of proliferation rates.

Instead, we focussed on an alternative approach (see also reviewer 3, point 4), which was to generate clones of fast-proliferating cells and measuring the consequences on neighbouring tissue, thus testing whether a local increase in growth rate is sufficient to induce tension in neighbouring slow-proliferating cells. We performed these experiments in the hinge region of the wing, where the results are not complicated by the "endogenous" tension gradient we observe in the pouch.

As shown in the new Fig. 6, when we induce mutant clones for the Hippo pathway component *warts (wts)*, which has been shown to result in tissue overgrowth, we observe considerable changes in neighbouring tissues:

- Image segmentation shows that cells around the clone become elongated perpendicular to the clone radius (Fig.6A-E).
- This alteration in cell elongation is correlated with increased tension in wild type cells along the clone border, as measured by laser ablation (Fig. 6F-H).
- Accordingly, cell divisions around the clones are also reoriented perpendicular to the clone radius (Fig. 6I-K).

Together, these data show that local overgrowth can induce neighbouring cell stretching and reorientation of cell division. This finding supports our model that the proliferation gradient observed in early wing imaginal discs (Fig. 7) acts as a driving force for proximal cell stretching and orientation of cell division perpendicular to the P-D axis.

3. Along the same vein, it is not clear if the observed tissue tension arises from the differential rates of division or if these rates of division only modulate pre-existing global tissue tension.

For example, in case there is no external tension, one might assume that there is 'negative' tension in the center where proliferation rates are higher than in proximal/marginal regions of the disc.

Since we can see the cells stretching already at 72 hours AEL before folds in the wing epithelium develop, it is unlikely that other global tissue tension patterns are affecting the stretching of circumferential cells. Although this is an interesting point, experimentally the system is too complex to cleanly dissociate global tension patterns from the effects of differential proliferation rates. This is precisely why the model is so valuable as it allows us to manipulate these factors and measure the outcome on cell behaviour. As pointed out in point 2 above, our new experiments suggest that differential growth can indeed drive increased tension.

Referee #2 (Remarks to the Author):

Here, they have looked more carefully at clone shapes and cell divisions in the wing disc, and report a slightly different finding: while growth is oriented towards the dv boundary in central regions of the wing pouch (as they previously found), it is oriented in a perpendicular direction in regions close to the hinge (i.e. growth is parallel to the hinge near the hinge).

The present manuscript does not conflict with our previous work (Mao *et al* Genes Dev 2011). In fact, we had noted the different orientation of growth at the periphery

of the pouch (Fig. S4) in the previous paper, and clearly stated that our analysis of division orientation was confined to the central region. The current work explains why these regional differences arise.

It is not clear how these findings relate to their previous model, which was that Dachs promotes growth along the PD axis by elongating cells in that direction. The authors argue in the discussion that Dachs may localize to cell boundaries in response to mechanical tension, and say that "this could allow the distal cells to continue to elongate and divide along the P-D axis whilst preventing the proximal cells from becoming excessively stretched, thus maintaining tissue integrity. The problem with this idea is that their quantifications of cell elongation in discs show that there is no region of the wing disc (also not in the middle) where cells are elongated parallel to the PD axis so their current experiments would seem to rule out this idea.

Dachs does bias the elongation of cells near the centre of the pouch (Fig 1G) but this bias is only apparent immediately **prior to mitosis** (which correlates with the cell division axis). At any 'steady state' (as in Fig. 3, S1), this P-D bias is disturbed by the fact that cells are at different stages of the cell cycle. The reason that the PD bias is not always apparent is firstly because the process of mitosis in this highly mitotic tissue yields two daughter cells that are not P-D polarized and will change shape over time. Secondly, it is probable that Dachs polarization is not uniformly transmitted to the daughter cells after mitosis, and therefore needs to be re-established over time. It is currently unclear how Dachs polarization changes during the cell cycle and to analyse this in depth would form the basis of a separate study. In addition, the focus of the present manuscript is not Dachs, but global tension patterns and proliferation rates, so these experiments would not add to the present manuscript.

The clear prediction of their model is that cells in *Fat* and *Dachs* mutant discs would be much more highly elongated parallel to the hinge throughout the wing pouch. It would have been easy to quantify cell shapes with their current methodologies in *Fat* and *Dachs* mutant discs, but this experiment hasn't been done.

We have shown that when Dachs polarization is lost (eg. In *fat* mutant wing discs) the cell shape immediately prior to mitosis is no longer P-D biased (Fig S2 and S3 in Mao et al 2011).

To further address this comment, we have examined cell shapes in whole *dachs* mutant discs (Referee Figure 1). As the referee anticipated, the proximal cells in this situation become extremely elongated parallel to the hinge. Because of the extreme nature of this phenotype, our automated segmentation software could not accurately identify the cell outlines to quantify cell shapes, but the result is very clear. We provide this as a referee figure but have not included it in the manuscript as this does not easily relate to the current work.

It is also hard to see how Dachs could localize to cell boundaries purely as a consequence of stress, when they and others have already shown that it localizes in response to the gradient of Dachsous expression. Unless they would like to argue that Fat and Dachsous affect the differential growth rate and only indirectly polarize Dachs through effects on growth. But none of this is discussed.

We still believe that the main cause of Dachs polarization is the Fat/Ds pathway. The referee refers to a statement in the discussion where we speculate on the possible stabilisation at junctions of Myo-II, and perhaps Dachs, due to increased tension. Although Fat/Dachsous is clearly the main signalling pathway responsible for Dachs polarisation, we were merely discussing the possibility that physical tension might reinforce Dachs localisation in P-D junctions of proximal cells, by analogy to Myo-II localisation in response to physical forces (Fernandez-Gonzalez *et al* Dev Cell 2009). As there is no evidence in the literature for Dachs localisation at junctions under tension, we have removed this statement (see p12).

The simulations also seem to indicate that cells are never elongated in the "PD" axis, at least if I understand Figure 6F correctly.

While the cell elongation ratio does decrease from the outside to the center of the field of cells, it is still greater than 1 in the center (I am assuming they are always measuring the elongation perpendicular to the PD axis - I can't find this information anywhere and if not, it should be explicitly stated).

An assumption in their vertex model is that cells would prefer to divide along their long axis, so how do clone shapes end up being parallel to the PD axis? Are there T1 transitions that are predicted by the model? If so, have the authors observed them in vivo? These things need to be more carefully explained.

We apologise if the text was unclear on this point. Figure 6F (which is now 7F) is the elongation ratio, rather than orientation (we now state that on p10). See Fig. S6 for elongation orientation, which is measured relative to PD (as indicated in the legend to Fig. S6). We have now made this more explicit in the legend ("orientation relative to P-D axis (0° is parallel to P-D and 90° is perpendicular to P-D)"). Therefore there is no inconsistency between P-D division orientation and P-D clone orientation.

Specific questions/points:

- While the authors present a very nice analysis of cell geometry, tissue organization and regional differences in proliferation rates over time, other analyses are not as carefully done with respect to time. Thus, it is not completely explained how the dynamics of growth actually work. Specifically:
- Live imaging: quantitation of cell division orientations was only done in the late discs (not actually specified, but since they mention hinge folds, it must be late).

As the referee states, the live imaging division orientation measurements were performed at ~100hr AEL. We now mention this in the methods (p14). Due to limitations in our culture conditions, it is not possible to do this analysis throughout disc development (see below).

- Boundary tension measurements: what stage of development was used for the laser ablation experiments? This is not mentioned, but seems vital to the argument that global forces are inducing anisotropies in cell elongation

These experiments were performed in 100hr AEL discs. We now state this in Figure 4 legend.

- Even better would be to have laser ablations over time to see when this difference arises and how that correlates to the evolution of anisotropies in cell area and polarization of phospho MyoII.

Currently we can only culture discs after 84h AEL. There is unlikely to be much difference between 84 and 120h since cell geometries do not change markedly during this time. Unfortunately we cannot keep 48h AEL discs alive to carry out laser ablations. In addition, the small size and fragility of young wing discs makes them unsuitable for live imaging. Konrad Basler's lab has encountered the same limitations of the explanted culture system, despite extensive optimization (Zartman *et al* Development 2013).

- Dachs polarization: I guess it is assumed to be present and polarized throughout growth, but previous papers have only looked at relatively late stages (?). When is Dachs polarization actually evident? Does it change over time? How does that relate to the transitions observed in tissue/cell geometry?

As requested by the referee, we have looked at Dachs polarisation in younger wing discs, and can observe it in second instar discs (Referee Figure 2). To measure temporal changes in Dachs localisation over a long period in this very mitotically active tissue would be a considerable challenge and would not advance the conclusions of this paper.

- In general, it needs to be explicitly written which experiments were live vs fixed and what stages were used for the analysis.

We have indicated this in the Figure legends.

- The paper would be much stronger with an analysis of cell shape and growth in the absence of Dachs (i.e, RNAi or mutant).

We have shown cell shape in Dachs depleted wing pouches (see above and Referee Figure 1). The present manuscript is not focussed on the role of Dachs in growth control, therefore an analysis of growth in Dachs mutants would not add to this work. We have looked at clone shape in *dachs* mutant clones in Mao *et al* 2011 (Figure 1).

Also, the simulations seem to already have Dachs built into them as an assumption - what happens if they take it out - how does it compare to Fat or Dachs mutants?

As suggested by the referee, we have performed *in vivo* mimicking simulations (as in the main manuscript Fig. 7K,L) with and without Dachs (Referee Figure 3). In the presence of Dachs, near the centre, clone orientations show a P-D axis alignment bias (median at 25° from the P-D axis) and similarly for division orientations (median 30°). When we remove Dachs from the simulations, this PD bias near the centre is lost, with both clone orientations and division orientations showing a random distribution (median around 45° from P-D axis). As expected, proximal cell divisions and clone orientations are still biased due to global forces. This supports the idea that Dachs participates in cell division orientation in the distal part of the disc.

- In Figure 2, I gather from the figure legend that these were fixed samples (since it is stated that the nubbin expression domain was used for selecting the analysis region). I'm not sure why they would use immunofluorescence when they have the ability to culture discs *ex vivo* and image live to watch how elongation and division angles are coordinated, as well as length of cell cycle.

At present we can only culture discs older than 84hrs and at best for 18hrs currently.

- Modeling:
- p7/Fig 5: why not also try introducing PD division axis bias in your model to see how this influences tissue/cell geometry?

We did simulations like this for the previous paper (results unpublished). This showed that when cells are elongated, even if cells are forced to divide to bisect the short axis (e.g. by forcing divisions along P-D even though they elongate perpendicular to P-D), daughter cells quickly realign to occupy the space of the mother cell, such that the end result is as if the cell divided to bisect the long axis.

- p7: When does the hinge fold develop (not obvious to those not studying *Drosophila* wing development)?

We have clarified in Figure 2 legend that this occurs at ~ 80 hr AEL.

From the images, best guess is between 72-96hr. AEL If the hinge fold were to be the origin of global forces acting on the whole pouch, it would have to be present before 72hr AEL, when the first anisotropies in area/elongation were noted. This point should be mentioned.

We agree with the referee that the folds are unlikely to cause the cell shape changes we observe and now mention this on p8).

- Were T1 transitions allowed in the model? Presumably yes, since they were in the last paper. Were they visualized in the live imaging? How many T1 transitions occur in the different scenarios (if any), and what happens if you don't allow rearrangements. Should be discussed.

The referee is correct, in that T1 transitions are allowed in the model. Because of the nature of the current model, where cells are not separate entities but share common sides, we cannot prevent T1 transitions. To address the referees comment we have used cell tracking in our movies of cultured wing discs in order to look at cell-cell rearrangement *in vivo* compared with *in silico* (Fig. S8). We find that, due to the technical limitations of the model, cell-cell rearrangements happen much faster *in silico* than *in vivo* (Fig. S8G).

- What happens in the different scenarios when Dachs is not included in the line tension?

This is already discussed in the previous page.

- Division orientation in simulated scenario that's most relevant to the *in vivo* scenario does not really seem to match experimental results. There is a much shallower difference in orientation of cell divisions along PD axis in the simulations compared with *in vivo*, even though the clone orientation agrees better (compare Fig 6J with 1F). This seems to contradict their model and suggest that additional elements are involved. It also suggests that T1 transitions contribute significantly to clone orientation in the simulations -is this correct? Again, does this occur *in vivo*?

As mentioned above, T1 transitions occur much more readily in the model than *in vivo*. This behaviour is expected to lead to a rapid loss of tension through cell rearrangements in the model. We believe this explains why, in *in silico* scenarios where we use the same mild proliferation gradient as observed *in vivo* (Figure 7) we see the same qualitative trend of the proximal cells to elongate and orient their divisions perpendicular to the P-D axis, but the model does not exactly quantitatively match the *in vivo* result because the system disperses tension more rapidly than *in vivo*. Yet despite this, we still observe a cell division bias in the *in vivo* mimicking scenario, supporting our hypothesis. Our aim in using the model is not to quantitatively reproduce the exact behaviour of the *in vivo* system, but to use it as a tool to qualitatively explore different scenarios. We now mention this issue in the discussion. In our new Figure 6, we also add further data supporting our model by showing that localised overgrowth can reorient neighbouring cell shape and division.

Finally, even in a perfectly P-D elongated clone, not all cells need to divide perfectly along the P-D axis, hence the cell division orientation trend will always be more shallow than the clone orientation trend, which is also reflected in the *in vivo* tissue (Fig 1D,F).

Minor points:

- p2: "Morphogenesis then further sculpts this post-mitotic tissue mass ...". The first two references following this statement clearly show that a tissue is NOT (necessarily) post-mitotic during morphogenesis (in fact the divisions are likely highly important for tissue shear). This is a classic error of confusing growth (increase in mass) with division (splitting of two cells), which can be separable.

We agree with the referee and have changed the text accordingly.

- p5: "At 48 hrs, the cells in the wing pouch are largely uniform in cell area and elongation...". Actually in the 48 hr timepoint, the error bars are by far the largest of all your timepoints, indicated a larger variability (not uniform) in cell area. You could say instead that there is less of a P-D bias in cell area, but don't saying that they are "largely uniform in cell area".

We have changed the text as suggested.

Indeed the drop in variation is probably meaningful, although SEM is not the correct measurement of variation in that case. What does the standard deviation look like?

We are not trying to show variability, but the P-D trends. Since 48h discs have very variable cell shapes, we have used SEM instead of S.D. such that it does not distract from our point, which is to show the P-D biases that emerge. We think the current representation of the data allows the reader to grasp the main concept of the paper.

- p5-6/Fig 3: The pattern of orientation actually does seem to change slightly between 96-120 around the center of the pouch.

This is likely to be due to "doming" of the disc as it grows, a process which will later be completed through the eversion process.

- p6: "This cell-autonomous anisotropy of Myo-II localization would be expected..."? How do you know it's cell-autonomous? Maybe the wording is confusing? Better to say "If the anisotropy in MyoII activity was cell-autonomous, it would be expected to ..."

We changed the text as suggested.

- p8: "Hence, the constriction ring is providing a physical force that does affect cell size ..." This is slightly misleading. You can say it is one possible solution, but this statement implies that this is the mechanism in vivo as well, which was not demonstrated.

We changed the text as suggested.

- Should probably address in the Discussion the biochemical-based models that have been proposed to explain how Dpp regulates even proliferation in the disc.

The role of morphogen gradients in growth control remains a very controversial field, which makes any discussion of this topic inconclusive. Our manuscript is not trying to explain why the proliferation rates are not uniform, but describes the outcome of this non-uniformity. We do discuss how our findings differ from previous models where growth is assumed to be uniform in the disc.

Figures:

Fig 1:

- It would be more useful to make B+C correspond to the example in A. Both in shape of overall pouch and in clones.

In B and C we deliberately used a different pouch shape to show that we did not simply split the disc in A into distal and proximal, but show an amalgamation of clones from several discs.

- Why are there no clones in the center?

Clones grow out from centre, so for a clone's centre, at end point, to be in the centre, it would have to cross the compartment boundaries, which they cannot do.

- In the particular examples of B+C, it appears that the elongation of the clones is less pronounced than in the distal center. Is that generally true?

The reviewer is correct. It is because the clones are smaller at the edges (further support for lower proliferation rate at edges) hence the extent of elongation is less pronounced.

In the measurements of clone orientation, is there any normalization for amount of clone elongation?

Only clones above a 1.25 elongation ratio are plotted to filter out the small minority of round clones. This will be stated in the figure legends.

- Likewise for cell elongation. Since cells in the center are less elongated, isn't it somewhat irrelevant for these cells? Showing orientation without extent of elongation is not as useful.

In Figure 1 legend we state only cells above ER of 1.3 are used in the analysis.

- Why not also absolute distance from the center? Not clear which is more important for the mechanics.

The wing disc is not a perfect circle, therefore relative distance is a more accurate way of representing the data.

Fig 2:

- Why don't you show nubbin expression, since this was (according to the legend) used to define the region of analysis (at least in young discs)?

We have now provided a *nub>GFP* figure in supplementary Fig. S2.

Fig 3:

- In part C, the legend says that the length of the bar indicates the extent of elongation, but all the bars look nearly the same length

The bars are not the same length, although zooming the image might be necessary to appreciate the differences as the figure is quite small.

Certainly the differences are far less obvious than the color coding in part B. Either add color or change the scale to include a higher dynamic range.

The bars are averaged over at least 10 cells, so ranges are less obvious than in individual cells (the outliers) in B.

- Also part C, it would be really useful if you could illustrate somehow (maybe with colored rings) where 0.1, 0.3, 0.5, 0.7, 0.9 "relative distances" are on this diagram so that it could more easily be compared to the plots of division orientation elsewhere in the paper. OR, even better, you could also plot cell elongation ratio and orientations in a way that makes it easier to compare to division orientation (i.e., also in a box+whisker plot as a function of distance from center).

These data are plotted in a graph that incorporates both the orientation and elongation ratios (Figure S3). In this situation, the data was divided into two bins (proximal and distal) otherwise cell number in the five individual bins (0.1, 0.3, 0.5, 0.7, 0.9) would be too low.

- In D+E, the error bars seem a bit too small. What does the standard deviation look like?

See above.

- Why are the data in D+E presented in line graphs? Better would be a scatter plot or box+whisker as the other data.

We have tried box plots but feel that box plots distract the reader from overall trends when dealing with noisy data like cell elongation *in vivo*.

Fig 5:

- Why is cell area so much more variable in situation 2 and 3 (looking at the images in part A)? (Again, this may be a reason to look at standard deviation rather than SEM).

Cells are dying in high friction situations (T2 transitions/delaminations). Again, we are not looking at variability but P-D trends.

Fig 6:

- Also show areas and elongation ratio for last simulated scenario (72hr as in I-K)

We have now provided this data (Fig. 7I, J).

- Part K: really difficult to see the patterns of clones. Can you make a diagram that looks more like the experiment, where not all cells are labeled (just some clones, rest white)?

We think it is more informative to be able to see a larger number of clones with multiple colours. The data are also plotted in Fig. 7K.

Can you quantify clone shape and size (as you did in last paper perhaps) and compare to experiment with respect to orientation along PD axis? That would make it easier to compare *in vivo* with *in silico* experiments.

We already have shape (orientation) plotted against P-D axis position (Fig. 7K, compare with *in vivo* Fig. 1D). Proliferation rates are parameterised in the model so will match the *in vivo* situation.

- For E+F see note above for Fig 3.

We have tried box plots but feel that box plots distract the reader from overall trends when dealing with noisy data.

Fig S2 is unnecessary

We think this figure provides an intermediate time point that describes the gradual change between 48 and 72 hrs, and is therefore useful.

Fig S3 should probably be in the main section of the paper.

This is not further explored in the paper so we think it would break the flow of the paper.

Methods:

- p14: in "fixed sample imaging" the 0.5-1mm intervals are probably meant to be in microns not mm.

We have fixed this.

- p14: what stage of discs were imaged and used to quantitate division angles?

100 hr AEL, we have now indicated this in the methods.

- What stage discs were used for the laser ablation experiments?

100 hr AEL, we have now indicated this in the methods.

Referee #3 (Remarks to the Author):

I have several major concerns, however, about the manuscript in its present form. These concerns should be addressed by modifications to the text and new experiments.

1. In the first results section on page 4, it is a bit unclear exactly which part of the presumptive wing is being analyzed. If it is only the central region, it would be helpful to know what fraction of the wing blade is actually generated from this central domain.

We apologise for not clearly specifying the region being analysed. We now provide a new Figure S1, which clarifies that area of interest at various stages.

Further, part of the idea here is that the deep tissue clefts surrounding the wing blade primordium constitute a physical barrier. How do rates of proliferation and orientations of division look within the folds? Although somewhat complex to analyze, it should be possible to compare the behavior of cells in the folds with those of the blade region. Are they subjected to a different set of forces as well? What fraction of the presumptive wing blade is actually generated from cells in the folds?

Although this is an interesting idea, this would represent a completely new story and would require considerable more time. The small size and convoluted nature of the wing fold in particular would make it very difficult to measure proliferation rates and cell topology reliably, as we were able to do in the pouch. We have clarified in the text that the wing blade is generated by the pouch, while the folds give rise to the hinge (see Fig. S1).

2. In the second results section beginning on page 5, the authors begin to use the term "epithelial topology" when they should be using the more appropriate term "epithelial geometry." Topology singularly refers to the global system of cell-neighbor relationships without taking into consideration apical surface areas, side lengths, elongation, cell volumes, etc. This could cause some confusion and should be revised throughout the paper.

We agree with the referee and have changed the text as requested.

3. On page 6, the authors should more clearly state the four distinct mechanisms since the way it's written initially makes it sound like two mechanisms.

We have re-written this part as requested (see p6).

" The proximal cells could either "autonomously" (1) extend their proximal/distal edges or (2) constrict their lateral edges (Fig. 4A, B). On the other hand, they could either (3) be compressed or (4) stretched by tissue-wide forces (Fig. 4A)."

In the analysis of ECad, Sqh, and p-Sqh in Fig S3, it would be helpful to see the localization relative to a uniform cortical or plasma membrane marker in addition to the individual channels.

We have performed a co-staining between E-cadherin and a membrane dye stain (FM4-64), which shows that E-cadherin is uniformly distributed around the plasma membrane (Fig. S5A-A''). We also provide merged images of E-cadherin with Sqh/Myosin light chain and p-Sqh, which illustrate the anisotropic p-Sqh localisation (Figure S5B-C'').

Could the authors explain more clearly how an external force might lead to localized p-Myo II localization?

As requested by the reviewer, we provide a more detailed discussion of the evidence supporting the reinforcement of the contractile actin network in response to tension on p12.

4. Without commenting on the modeling itself, the authors use in silico methods to validate their general hypothesis- which is that spatially differential growth rates lead to corresponding spatial patterns of cell elongation and division orientation in the wing disc. In order to more directly explore this idea, the authors should generate local changes in proliferation rate in order to experimentally validate any corresponding local effects on cell division orientation. This could be done either by locally perturbing rates of cell proliferation directly, or by altering spatial patterning cues that set up the p-d axis and then examining the effects on cell division orientation.

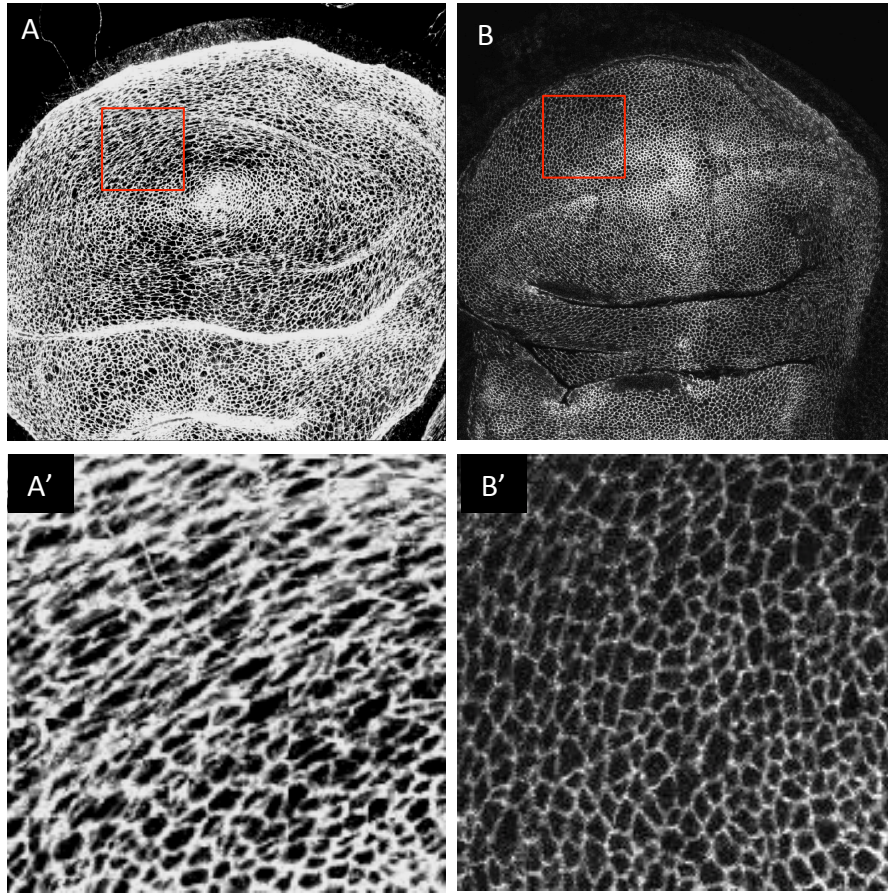
As suggested by the referee, we generated clones of fast-proliferating cells and measured the consequences on neighbouring tissue, thus testing whether a local increase in growth rate is sufficient to induce tension in neighbouring slow-proliferating cells. We performed these experiments in the hinge region of the wing, where the results are not complicated by the "endogenous" tension gradient we observe in the pouch.

As shown in the new Fig. 6, when we induce mutant clones for the Hippo pathway component *warts* (*wts*), which is known to induce tissue overgrowth, we observe considerable changes in neighbouring tissues:

- Image segmentation shows that cells around the clone become elongated perpendicular to the clone radius (Fig.6A-E).
- This alteration in cell elongation is correlated with increased tension in wild type cells along the clone border, as measured by laser ablation (Fig. 6F-H).
- Accordingly, cell divisions around the clones are also reoriented perpendicular to the clone radius (Fig. 6I-K).

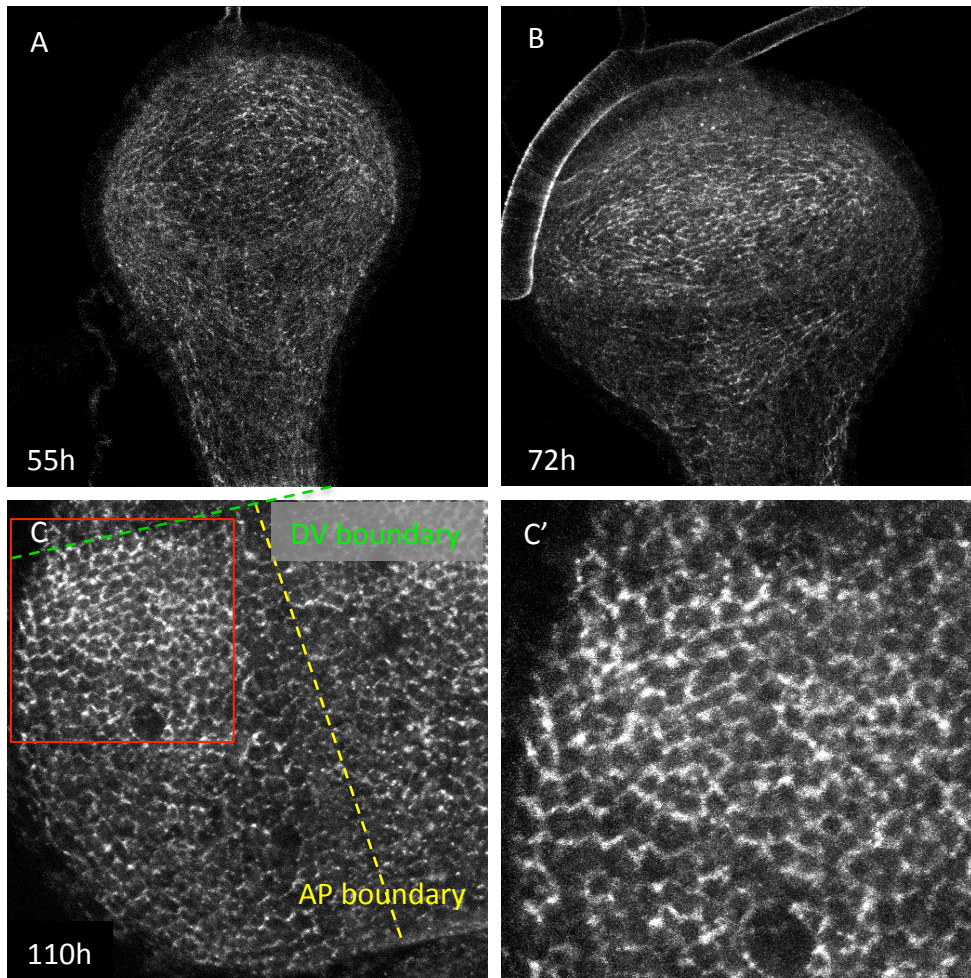
Together, these data show that local overgrowth can induce neighbouring cell stretching and reorientation of cell division. This finding supports our model that the proliferation gradient observed in early wing imaginal discs (Fig. 7) acts as a driving force for proximal cell stretching and orientation of cell division perpendicular to the P-D axis.

Mao_Referee Figure 1



(A-A') *NubGal4>DachsRNAi* wing discs stained with anti-E-cadherin. Cells are clearly stretched more circumferentially compared to WT wing disc cells **(B)**. This is consistent with Dachs increasing junctional tension on the P/D junctions which would resist the global circumferential stretching force of the growing tissue, especially near the periphery. As the cells were so distorted (long and thin) our automated image segmentation software could not accurately segment individual cell outlines for us to generate quantitative data, to compare with WT cells.

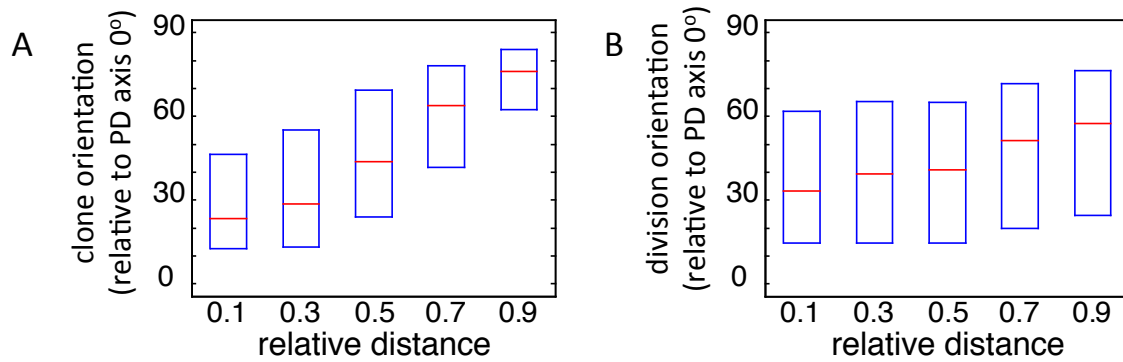
Mao_Referee Figure 2



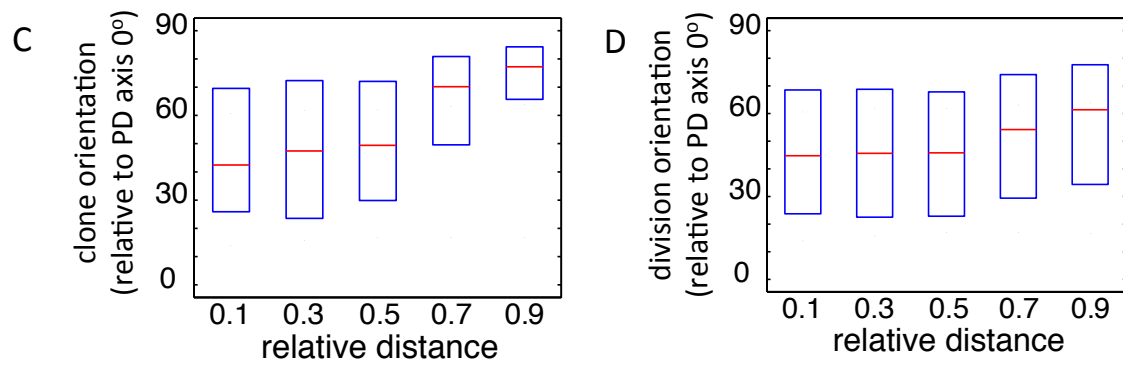
(A-B) Using a Dachs::GFP line (kind gift from Yohanns Bellaïche) we were able to detect Dachs polarisation in young wing discs from about 55h AEL. **(C-C')** In older wing discs we could detect Dachs polarisation in the centre of the pouch when imaged at high magnification.

Mao_Referee Figure 3

With Dachs



Without Dachs



(A-B) *In vivo* mimicking simulations with Dachs as in the main manuscript Fig. 7K,L. Near the centre, the clone orientations show a PD axis alignment bias (median at 25° from the PD axis) and similarly for division orientations (median at 30°). **(C-D)** When we remove Dachs from the simulations, this PD bias near the centre is lost, with both clone orientations and division orientations showing a random distribution (median around 45° from PD axis).

Thank you again for the submission of your revised manuscript to The EMBO Journal and please accept my apologies for the delay in our response, due to the holiday season and the difficulty to contact referees this time of the year. Your study was sent back to one of the original reviewers, who now considers that most of his/her major concerns have been properly addressed and your manuscript is almost ready for publication.

As you will see below, referee #1 still point out to two related issues regarding your clonal analysis that need your attention before your manuscript can be accepted. Browsing through the manuscript myself, I have also noticed that some of your micrographs lack scale bars, which we require for clarity.

Thank you very much again for your patience. Once these minor issues have been addressed, I will be glad to accept your manuscript for publication.

I am looking forward to seeing the revised, final version of your manuscript.

REFEREE REPORTS

Referee #1

Mao and colleagues have satisfactorily addressed most of the points raised during the first round of review, and - as a result of this - the manuscript has considerably improved. Nevertheless, there are some points that should still be addressed.

In an attempt to understand *in vivo* how differential proliferation rates can influence tissue tension and cell geometry/division orientation, the authors generated clones of fast-proliferating cells within the hinge region of the wing, and analyzed the effects caused in the slower proliferating cells surrounding these clones.

1) While the observation that cell junctions around the clone borders display higher tension than comparable junctions in control hinges w/o clones is interesting, the authors still need to demonstrate that tension anisotropy is increased in the tissue surrounding those clones. In the end it's tension anisotropy that determines the cell division orientation.

2) It might also be interesting to test what predictions would arise from applying their model to the new clonal approach and how far those predictions correspond to the experimental results. This might be particularly interesting, as it would allow testing how far the model predictions depend on the specific assay system used (clones vs no clones; hinge vs blade).

Minor point

In page 6 the authors write: "Using laser ablation to measure junctional tension..."

By performing laser ablation one measures recoil velocities, which can be translated into tension depending on the specific viscoelastic properties of the tested tissue. Better might be to say '... to reveal junctional tension ...'.

We have addressed referee #1's comment as detailed below.

1) While the observation that cell junctions around the clone borders display higher tension than comparable junctions in control hinges w/o clones is interesting, the authors still need to demonstrate that tension anisotropy is increased in the tissue surrounding those clones. In the end it's tension anisotropy that determines the cell division orientation.

As requested by the reviewer, we have ablated the junctions perpendicular to the clone border (radial junctions) and present the results in Fig. 6G-H. Interestingly, these junctions are under less

tension than even control junctions, supporting the idea that cells around the periphery of an overgrowing clone become stretched circumferentially, leading to reorientation of cell division due to force anisotropy.

2) It might also be interesting to test what predictions would arise from applying their model to the new clonal approach and how far those predictions correspond to the experimental results. This might be particularly interesting, as it would allow testing how far the model predictions depend on the specific assay system used (clones vs no clones; hinge vs blade).

As requested, we have added a model scenario to mimick overgrowing clones in Fig. 6L-O. As in the *in vivo* situation, the cells around the clone become stretched circumferentially.

Minor point

In page 6 the authors write: "Using laser ablation to measure junctional tension..."

By performing laser ablation one measures recoil velocities, which can be translated into tension depending on the specific viscoelastic properties of the tested tissue. Better might be to say '... to reveal junctional tension ...'.

We have corrected this as suggested.