Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Cell fate coordinates mechano-osmotic forces in intestinal crypt formation.
Corresponding author name(s): Prisca Liberali

Reviewer Comments & Decisions:

Decision Letter, initial version:

Date: 29th September 20 11:49:13
Last Sent: 29th September 20 11:49:13
Triggered By: Christine Weber
From: christine.weber@nature.com
To: prisca.liberali@fmi.ch
CC: ncb@springernature.com
Subject: Decision on Nature Cell Biology submission NCB-L43714-T
Message: *Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Prisca,

As Dr Andrew Cox has now left the journal, I will be taking over the handling of your paper from him. I am writing to let you know that your manuscript, "Cell fate coordinates mechano-osmotic forces in intestinal crypt formation", has now been seen by 3 referees, who are experts in intestinal organoids, mechanobiology (referee 1); intestinal organoids (referee 2); and biomechanics, morphogenesis, modelling (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to address the following editorial priorities:

(A) Additional mechanistic insights should be provided, as highlighted by referees 1 and 2.

Referee 2 notes:

“As a mechanism, the authors explore differential localization of ZO1, Occludin, Claudin2 and N-cadherin. They observe interesting crypt-specific patterns of Occludin, Claudin2 and N-cadherin while Z01 was expressed in the crypt and villus apical surface and in the villus basal surface. They conclude “However, among them, only ZO-1 overlaps with the basal pool of Myh-9-GFP in organoid villus region, and exhibits villus basolateral localization both in vitro and in vivo during crypt morphogenesis”. Yet, the authors do not provide in vivo data for Occludin, Claudin2 or N-cadherin. Moreover, they did not look at any of the markers with high temporal resolution around the time of crypt emergence in vivo (i.e. around Post-natal day 14). It is possible that both basal Z01 in the villus region coupled with apical Occludin/Claudin2/N-cadherin in the crypt domain are both important for the process of actomyosin patterning and crypt budding. It seems that the conclusion that Z01 contributes to the emergence of the region-specific actomyosin pattern overlooks this possibility. Some additional immunostaining in vivo during the time of crypt formation will be helpful to add this valuable information.”

Referee 1 notes:

“5. Fig. 2E and F: To be able to draw conclusions from the difference of tension in the crypt and villus region, the authors should verify the correlation between Myh9 expression and the intensity, as it seems like the intensity is higher where there is a curvature which could be due to the compactness of the cells in those regions.”

“7. In Fig. S5C, the enrichment of villus basal Myh9 is not clear. Removing the signal from the dead cells may enhance the contrast.”

“16. How do the ion channel piezo1 or piezo2 as mechanosensors affect the emergence of bulge or bud in the organoids? Experiments should be conducted using an activator (Yoda-1) or inhibitor (GdCl3) of these piezo channels.”

(B) Additional experiments should be performed to confirm that lumen volume reduction accelerates crypt morphogenesis and it should be tested whether additional factors could have an effect, as pointed out by all three referees.

Referee 1 notes:

“11. In Fig. S8E, the authors aimed to show the effect of deflation using an osmotic shock on crypt emergence; however, the red arrows are not convincingly showing any emergence of crypts.

12. In Fig. 4, the authors should explain how they have derived enterocysts. Moreover, it is not clear how the collapse of the inflated epithelium is related to the increase of enterocyte size. For example, luminal fluid could escape through the rupture of the epithelial monolayer or just go into a different part of the organoid, or even through paracellular leakage."

Referee 2 notes:

"For lumen volume experiments that used osmotic shock – the authors should show that cells are viable and that the cells themselves are not affected by the osmotic shock. Should the osmotic shock affect the cells themselves (and not just lumen volume), the current interpretation of the experiment would likely need to be revised.
Also – for these experiments, the authors concluded "seconds after the osmotic deflation, the Day3.5 organoids formed bulges in the crypt regions enriched in Lgr-5+ stem cells, while the Day2.5 and Day3.5 CHIR-treated organoids remained spherical and did not display significant bulging"; however, in the eccentricity measurement, the day 3.5 CHIR showed statistically significant increased eccentricity after osmotic shock (Fig 8SE – box plot). Is this conclusion at odds with the statistical analysis?

Figure 4D – are the authors claiming that total enterocyst volume does not change? It seems that lumen volume goes down, cell volume does go up, but total volume goes up initially and then back down. Can the total loss of lumen volume be accounted for by the increase in cell volume? It does not seem like cell volume increases sufficiently to account for 100% of lumen volume loss, but this is something the authors could calculate."

"The authors nicely show using LifeAct and Myh-9-GFP, coupled with direct measurements of the tension in the basal surface of crypts vs. villi that there are differential tensions and localization of Myh-9-GFP on the basal surface. Assuming that the epithelium of both the crypt and villus region are interacting with the homogeneous extracellular matrix (Matrigel) during the process of crypt budding, have the authors examined the interaction of the crypt and villus epithelium with the surrounding matrix, and is there any evidence that this interaction (i.e. remodeling of matrix proteins) is correlated with the crypt region vs. the villus region? That is, is the matrix also an active participant in this process, or a passive bystander that simply gets pushed around by epithelial forces?"

"For Figure 4A – were enterocysts and CHIR grown organoids given osmotic shock? The section heading indicates this is the case, but the text and figure legend do not explicitly state they were treated with osmotic shock."

Referee 3 notes:

• Lumen shrinkage and epithelial volume increase are nicely demonstrated. However, the authors do not discuss the possibility of local differences in cell division contributing to the crypt vs. villus epithelium differentially. Are there local differences in cell division and if yes, how would they affect the model?

• The authors note that the mesenchyme is important for the formation of villi in vivo and say that the organoids can form a crypt and villus without mesenchyme. Could the authors discuss possible effects of the mesenchyme on crypt morphogenesis in vivo, especially how it would impact the mechanical landscape? This would be helpful and contextualize the work better in existing work on intestinal development (e.g. references 5, 11).

• The authors propose that osmotic transport of fluid into the enterocytes in the villus results in lumen volume reduction. In Supplementary Figure 9, they track crypt and villus cell volume changes. A figure showing the volume reduction in the lumen side by side with the total volume increase in the villus epithelium (or a similar comparison) would strengthen this point and add additional quantification to it.

(C) The association of cell fate and osmotic and actomyosin forces in intestinal crypt formation should be further investigated to avoid overstatements.

Referee 2 notes:

"Is the differentiation of a Paneth cell at all correlated with apical constriction and crypt formation? Would crypts/buds form in the absence of PCs, such as in the ATOH1-null epithelium? Certainly crypts seem to form just fine in genetic ATOH1-null animals; however since this group has attributed the symmetry breaking (and initiation of bulging/budding) of the cyst in part to PCs, it would be interesting to know if PC are in any way correlated to Myh-9-GFP in the context of organoid crypt budding."

Referee 3 notes:

"• The title of the paper comprises the term "cell fate coordinates", however how cell fate leads to differential apical actin constriction in the crypt region is not a major focus of the paper. I suggest to either include more data on how cell fate controls apical constriction and thereby tissue curvature or rephrase the title to better represent the focus of the paper on apical/basal tension, tissue curvature, lumen volume and cell swelling."

(D) All other referee concerns pertaining to increasing sample sizes (at least 3 per experiment), strengthening existing data, providing further methodological clarifications and textual changes should also be addressed. Please ensure that figure legends do not exceed 350 words - all descriptions of findings should go into the Results section.

(E) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Reporting Summary (found here https://www.nature.com/documents/nr-reporting-summary.pdf). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

https://mts-ncb.nature.com/cgi-bin/main.plex?el=A6C1CuG2A1umA1J4A9ftdNQ3TtDf0VWaw1n6fd7OJwZ

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months. We are aware that many researchers are currently facing disruptions because of the COVID-19 pandemic. If you anticipate significant delays for these revisions, please do let us know as we are happy to extend deadlines as necessary.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

With best wishes,

Christine.

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, Yang et al explore the mechanisms of mouse intestinal crypt morphogenesis by developing a 3D vertex model and combining it with light-sheet microscopy. The authors demonstrate that in addition to actomyosin contraction, lumen volume reduction via cell swelling in the villus region is crucial for crypt formation. This work highlights the role of mechano-osmotic forces during crypt morphogenesis and is of interest to the organoid and mechanobiology community. There are several concerns that need to be addressed to render the manuscript acceptable for publication:

1. In Figure 1B, and C (and Fig. S9B), it is not clear how the segmentation was done for the crypt section of the bulged organoids.

2. Statistical analysis for Figure 1C needs to be repeated considering n = number of the organoids, not single cells or preferably organoids from different time points. P values of 10^-36 or -28 have been derived based on an incorrect “n”.

3. In Fig. S1A, the reduction of distance between adjacent nuclei (as mentioned in line 85 of the text) cannot be seen by immunostaining. It seems that the distance has been increased. Counting the number of nuclei in the crypt area may be a better readout here. Also, y-axis labels of Figure S1A' and B' are missing. These findings are also in contradiction to the schematic of Fig. 1A. Fig. S1 C, D are not clearly showing the conservation of the observed phenomenon in vivo.

4. Model derivation:
* line 312: does it mean that 2
* line 324: equation 6 is somewhat misleading as after derivation the substituted boundary conditions (R at equilibrium) and the variable radius have been labeled similarly (also in line 325 while defining the deformation ratio). The same is true for equation 7.

5. Fig. 2E and F: To be able to draw conclusions from the difference of tension in the crypt and villus region, the authors should verify the correlation between Myh9 expression and the intensity, as it seems like the intensity is higher where there is a curvature which could be due to the compactness of the cells in those regions.

6. Please discuss the paper by Zhao et al. Nat. Comm. 2015 on the effect of Blebbistatin in crypt formation regarding Fig. S5. In this paper Blebbistatin was shown to enhance crypt formation in mouse intestinal organoids.

7. In Fig. S5C, the enrichment of villus basal Myh9 is not clear. Removing the signal from the dead cells may enhance the contrast.

8. In Fig. S5D, n=2 is insufficient (line 1125).

9. In the caption of Fig. S5 (line 1129), it should be re-growth instead of re-grow.

10. Fig. S8D,E: How does the signal of Lgr5 change after inflation and collapse/deflation of the organoids?

11. In Fig. S8E, the authors aimed to show the effect of deflation using an osmotic shock on crypt emergence; however, the red arrows are not convincingly showing any emergence of crypts.

12. In Fig. 4, the authors should explain how they have derived enterocysts. Moreover, it is not clear how the collapse of the inflated epithelium is related to the increase of enterocyte size. For example, luminal fluid could escape through the rupture of the epithelial monolayer or just go into a different part of the organoid, or even through paracellular leakage.

13. Line 305 of the main text refers to Fig. S9D and not Fig. S9C.

14. Line 314 of the main text refers to Fig. 4H.

15. Moreover, line 316 and 318 refer to Fig. 4I-I' and S9E instead of Fig.4H and Fig.S9D.

16. How do the ion channel piezo1 or piezo2 as mechanosensors affect the

emergence of bulge or bud in the organoids? Experiments should be conducted using an activator (Yoda-1) or inhibitor (GdCl3) of these piezo channels.

Reviewer #2:
Remarks to the Author:
Yang and Xue and colleagues follow up on previous work from the Liberali lab that showed how intestinal organoids/enteroids break symmetry after initially forming as a uniform cystic structure. In the current work, the authors focus on the cellular mechanisms that drive the process of bulging and budding during crypt formation in organoids following this symmetry breaking event. They demonstrate that apical contraction in crypts and basal tension in the villus generate curvatures leading to crypt formation, and that enterocytes contribute to this process by swelling.

Overall this is a strong manuscript. It validates qualitative observations with rigorous quantitative analysis, and also provides mathematical modeling and theory to describe the observations being made. Thus, claims made are mostly supported with compelling data.

I had only a handful of comments/critiques/questions:

Major points:

Is the differentiation of a Paneth cell at all correlated with apical constriction and crypt formation? Would crypts/buds form in the absence of PCs, such as in the ATOH1-null epithelium? Certainly crypts seem to form just fine in genetic ATOH1-null animals; however since this group has attributed the symmetry breaking (and initiation of bulging/budding) of the cyst in part to PCs, it would be interesting to know if PC are in any way correlated to Myh-9-GFP in the context of organoid crypt budding.

As a mechanism, the authors explore differential localization of ZO1, Occludin, Claudin2 and N-cadherin. They observe interesting crypt-specific patterns of Occludin, Claudin2 and N-cadherin while Z01 was expressed in the crypt and villus apical surface and in the villus basal surface. They conclude “However, among them, only ZO-1 overlaps with the basal pool of Myh-9-GFP in organoid villus region, and exhibits villus basolateral localization both in vitro and in vivo during crypt morphogenesis”. Yet, the authors do not provide in vivo data for Occludin, Claudin2 or N-cadherin. Moreover, they did not look at any of the markers with high temporal resolution around the time of crypt emergence in vivo (i.e. around Post-natal day 14). It is possible that both basal Z01 in the villus region coupled with apical Occludin/Claudin2/N-cadherin in the crypt domain are both important for the process of actomyosin patterning and crypt budding. It seems that the conclusion that Z01 contributes to the emergence of the region-specific actomyosin pattern overlooks this possibility. Some additional immunostaining in vivo during the time of crypt formation will be helpful to add this valuable information.

For lumen volume experiments that used osmotic shock – the authors should show that cells are viable and that the cells themselves are not affected by the osmotic shock. Should the osmotic shock affect the cells themselves (and not just lumen

volume), the current interpretation of the experiment would likely need to be revised.
Also – for these experiments, the authors concluded "seconds after the osmotic deflation, the Day3.5 organoids formed bulges in the crypt regions enriched in Lgr-5+ stem cells, while the Day2.5 and Day3.5 CHIR-treated organoids remained spherical and did not display significant bulging"; however, in the eccentricity measurement, the day 3.5 CHIR showed statistically significant increased eccentricity after osmotic shock (Fig 8SE – box plot). Is this conclusion at odds with the statistical analysis?

Figure 4D – are the authors claiming that total enterocyst volume does not change? It seems that lumen volume goes down, cell volume does go up, but total volume goes up initially and then back down. Can the total loss of lumen volume be accounted for by the increase in cell volume? It does not seem like cell volume increases sufficiently to account for 100% of lumen volume loss, but this is something the authors could calculate.

Minor points:

Perhaps semantics or differences of word usage in different fields (i.e. biological vs. mathematical/theoretical), but the authors refer to the change in curvatures that lead to crypt formation as "spontaneous"; however, their previous work (Serra and Mayr et al.) demonstrated that the process of symmetry breaking is very stereotyped and reproducible. That is, the formation/differentiation of a Paneth cell preceeds budding. Thus, the word "spontaneous" seems rather at odds with an active and stereotyped process in the biological sense.

The authors nicely show using LifeAct and Myh-9-GFP, coupled with direct measurements of the tension in the basal surface of crypts vs. villi that there are differential tensions and localization of Myh-9-GFP on the basal surface. Assuming that the epithelium of both the crypt and villus region are interacting with the homogeneous extracellular matrix (Matrigel) during the process of crypt budding, have the authors examined the interaction of the crypt and villus epithelium with the surrounding matrix, and is there any evidence that this interaction (i.e. remodeling of matrix proteins) is correlated with the crypt region vs. the villus region? That is, is the matrix also an active participant in this process, or a passive bystander that simply gets pushed around by epithelial forces?

In Figure 2B, it will be helpful if the authors label each of the 3 scenarios in the schematic corresponding to their numerical assignments in the text (i.e. scenario "i)", "ii)", and "iii)").

In the text the authors state: "This provides a key qualitative test of the mechanism we propose: a mechanism of softer crypts would result in the reverse trend of preferential crypt expansion, a mechanism of budding via crypt cell proliferation (i.e. buckling) would result in crypt opening as fluid injection increases the area/volume ratio (22) (see SI Text for detailed discussion)." Here, the authors state two alternative possibilities to the mechanism they propose. It will be helpful for the readers if they re-state their proposed mechanism first, and explicitly call the discussion points above "alternative mechanisms", which do not fit their model.

For Figure 4A – were enterocysts and CHIR grown organoids given osmotic shock? The section heading indicates this is the case, but the text and figure legend do not explicitly state they were treated with osmotic shock.

Figure 4 panels are mis-labeled in the text (i.e. figure 4G).

Reviewer #3:
Remarks to the Author:
Summary:
In this study, the authors use a 3D organoid culture model to identify a mechanical mechanism for crypt morphogenesis. The authors examine the effects of actomyosin-driven apical contraction, villus basal tension, lumen volume and tissue volume on the geometry of organoids with developing intestinal crypts both experimentally and in a biophysical model. The authors nicely use their model to guide experimental perturbations that are then used to confirm model predictions and find that differential spontaneous curvature in the crypt vs. villus region together with lumen volume reduction can explain the morphological changes during crypt budding. The differential curvature nicely matches the pattern of myosin localization at the apical side of the bulging crypt and at the basal side of the villus region leading to increased tension as demonstrated by laser nanosurgery and micropipette aspiration assays. Upon inflation of the lumen using pharmaceutical and mechanical methods, budded crypts could not be opened, but less developed bulged crypts could be as predicted by the model. Furthermore, lumen volume was found to be osmotically redistributed from the lumen to the enterocytes in the villus region, increasing compressive stress on the crypt and thereby supporting budding. The authors demonstrated that although the geometry in vivo has an open lumen, swelling of cells in the villus could still contribute to crypt morphogenesis in vivo. In conclusion, the authors elegantly overcome the difficulty of studying an internal organ with limited accessibility by taking advantage of live imaging of intestinal organoids in combination with biophysical modeling to study the mechanical mechanism of crypt formation.

Major Points:
• The title of the paper comprises the term “cell fate coordinates”, however how cell fate leads to differential apical actin constriction in the crypt region is not a major focus of the paper. I suggest to either include more data on how cell fate controls apical constriction and thereby tissue curvature or rephrase the title to better represent the focus of the paper on apical/basal tension, tissue curvature, lumen volume and cell swelling.
• Lumen shrinkage and epithelial volume increase are nicely demonstrated. However, the authors do not discuss the possibility of local differences in cell division contributing to the crypt vs. villus epithelium differentially. Are there local differences in cell division and if yes, how would they affect the model?
• The authors note that the mesenchyme is important for the formation of villi in vivo and say that the organoids can form a crypt and villus without mesenchyme. Could the authors discuss possible effects of the mesenchyme on crypt morphogenesis in vivo, especially how it would impact the mechanical landscape? This would be helpful and contextualize the work better in existing work on intestinal development (e.g. references 5, 11).

Minor Points:

• The term “spontaneous curvature” is central to the manuscript and should be explained to readers who are not familiar with this terminology. The term “spontaneous curvature” should be clearly separated from the term “spontaneous symmetry breaking” to prevent misunderstandings. If I understand correctly, cell fate leads to local differences in tissue curvature and crypts do not form because of a spontaneous local increase in curvature at a random position of the spheroid.
• One of the major points in the paper is that the spontaneous curvature of the tissue drives morphogenesis. However, the curvature of the villus epithelium in the organoids is inverted compared to the in vivo situation. Could the authors comment/discuss this point?
• The authors propose that osmotic transport of fluid into the enterocytes in the villus results in lumen volume reduction. In Supplementary Figure 9, they track crypt and villus cell volume changes. A figure showing the volume reduction in the lumen side by side with the total volume increase in the villus epithelium (or a similar comparison) would strengthen this point and add additional quantification to it.
• Ref. 11 talks about a “hinge” between the villus and crypt. When the authors discuss in vivo applications of their findings, incorporating a short comment on effects the hinge region would have on morphogenesis would be helpful.
• Figure 2D: The figure panels are very small. Please increase the size by using empty white space in the figure to allow the reader to see the nanosurgery experiments.
• Figure 2D’’: The error bars overlap and are not easy to read. Incorporating a design similar to Fig. 1E would improve readability.
• Figure 2E: Include a panel showing a zoom-in onto the micropipette so that the reader can see the difference in basal tension in the crypt vs vilus region.
• Figure 2F, F’, F’’, F’’’: The authors show an image of basal Myh-9-GFP intensity in the villus and crypt, but do not quantify or directly compare the two, instead incorporating them into an overall intensity in F’’. A figure similar to F’ but for the basal side would be helpful to compare myosin between the two regions on the basal side (presumably important for maintaining villus tension).
• Figure 2 legend: The legend is very long. Consider shortening for example by moving information about method details (for the micropipette aspiration assay) to the method section rather than the figure legend.
• Methods section: please make sure to explain abbreviations used such as FCN
• Typos in the text:
o Line 61: “Day3” and “Day4” are missing blanks
o Line 137: There is no Fig. 2C’’’. Please refer to the correct figure panel.
o Line 151: Fig. 2D-D’’ should read Fig. 2D-D’’’.
o Line 314: “Fig. 4G” should be replaced with “Fig. 4H”.
o Line 318: “Fig. 4H” should be replaced with “Fig. 4I”.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

MANUSCRIPT FORMAT – please follow the guidelines listed in our Guide to Authors regarding manuscript formats at Nature Cell Biology.

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see https://www.nature.com/licenceforms/nrg/competing-interests.pdf.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled “Statistics and Reproducibility”.

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement as a separate section after Methods but before references, under the heading "Data Availability”. . For Springer Nature policies on data availability see http://www.nature.com/authors/policies/availability.html; for more information on this particular policy see http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf. The Data availability statement should include:

• Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here http://www.nature.com/authors/policies/availability.html#data.

• Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details http://www.nature.com/sdata/data-policies/repositories).

• At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

• If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. More details can found at www.nature.com/protocolexchange/about.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs $600 for the first, and $300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend.
Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.

- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the "unprocessed scans" Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – We are trying to improve the quality of methods and statistics reporting in our papers. To that end, we are now asking authors to complete a reporting summary that collects information on experimental design and reagents. The Reporting Summary can be found here https://www.nature.com/documents/nr-reporting-summary.pdf)If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.

Author Rebuttal to Initial comments

Reviewer #1

Remarks to the Author:

In this manuscript, Yang et al explore the mechanisms of mouse intestinal crypt morphogenesis by developing a 3D vertex model and combining it with light-sheet microscopy. The authors demonstrate that in addition to actomyosin contraction, lumen volume reduction via cell swelling in the villus region is crucial for crypt formation. This work highlights the role of mechano-osmotic forces during crypt morphogenesis and is of interest to the organoid and mechanobiology community. There are several concerns that need to be addressed to render the manuscript acceptable for publication:

1. In Figure 1B, and C (and Fig. S9B), it is not clear how the segmentation was done for the crypt section of the bulged organoids.

The crypt regions in bulged and budded organoid were selected depending on the crypt morphology. In the revised manuscript, we further validated our region selection with previously performed Lysozyme staining for Paneth cells. The end point of crypt was chosen as the cells that were in the regions of bulged or budded curvature and with Lysozyme positive cells. An example of how we selected the crypt region in bulged organoid is now demonstrated in Fig. S1A, and the details are added in the method section.

2. Statistical analysis for Figure 1C needs to be repeated considering n = number of the organoids, not single cells or preferably organoids from different time points. P values of 10^-36 or -28 have been derived based on an incorrect "n".

In the revised manuscript, we have increased the number of organoids for each group (before, bulged and budded) to 9 Day3,7 Day3.5 and 6 Day4. Since the plot is showing the data from single cells, the P-values are generated from the same data. We have added new data to Fig. 1C with newly calculated P-values for: 1) all the single cells and 2) the average values for each organoid in figure legend of Fig. 1.

3. In Fig. S1A, the reduction of distance between adjacent nuclei (as mentioned in line 85 of the text) cannot be seen by immunostaining. It seems that the distance has been increased. Counting the number of nuclei in the crypt area may be a better readout here. Also, y-axis labels of Figure S1A' and B' are missing. These findings are also in contradiction to the schematic of Fig. 1A. Fig. S1 C, D are not clearly showing the conservation of the observed phenomenon in vivo.?

We thank the reviewer for the suggestion. To better display the nuclei, we have now changed the colour of staining. In addition, we performed a new analysis of counting nuclei in the crypt/villus regions and measured the cell density along the crypt-villus axis. The new data is now displayed in Fig. S1 B-C'. The result shows crypt tissue compaction is conserved in organoids and *in vivo* tissue.

Now the new y-axis labels (Cell density (Cell No. / 10 um)) are added.

4. Model derivation:

* line 312: does it mean that $2<R/h<10$? If so, please add a reference for that.
* line 324: equation 6 is somewhat misleading as after derivation the substituted boundary conditions (R at equilibrium) and the variable radius have been labeled similarly (also in line 325 while defining the deformation ratio). The same is true for equation 7.

On the first point, this was based on our own measurement of organoid aspect ratio, which we had already performed for the fitting of the morphogenetic evolution of organoids (Fig. 2) and their behaviour upon lumen inflation (Fig. 3). Indeed, in these measurements, we constrain the model by independently measuring the geometrical parameters of the system such as crypt fraction or rescaled initial thickness of the organoid. This was mentioned in section 4.2.1 (as the values of shape factor k0, which is related to h/R), but we realize that this nomenclature was unclear. We now refer directly to the derivation of the model to better clarify the validity of the assumptions (lines 994-998, page 33 in supplementary). On the second point, this was indeed a typo introduced in the conversion of the equation, and was making things unclear, we thank the referee for spotting this and have clarified the annotations in Eq. 6 and 7 (line 362 and 379 page 11 in supplementary).

5. Fig. 2E and F: To be able to draw conclusions from the difference of tension in the crypt and villus region, the authors should verify the correlation between Myh9 expression and the intensity, as it seems like the intensity is higher where there is a curvature which could be due to the compactness of the cells in those regions.

We thank the reviewer for this point. To normalize any compaction-related increase of Myh-9-GFP intensity, we have now measured the intensity of the membrane-targeted green fluorescent protein (mG) (*1*)and compared its expression with Myh-9-GFP (Fig. S5E). In opposition to the Myosin expression pattern, the data shows that the intensity of mG at apical side is higher in villus than in crypt (which is due to the development of microvilli in villus tissue). Less mG but more Myh-9-GFP intensity at the crypt apical side indicates that the enrichment of Myh-9-GFP there is independent of tissue compaction during bulging and budding.

6. Please discuss the paper by Zhao et al. Nat. Comm. 2015 on the effect of Blebbistatin in crypt formation regarding Fig. S5. In this paper Blebbistatin was shown to enhance crypt formation in mouse intestinal organoids.

We thank reviewer for this question. The paper published by Zhao *et al.* (2015) is a very nice paper where they characterize the role of Myosin IIA (Myh-9) during intestinal regeneration and they perform many experiments in organoids (*2*) . Most of their experiments is, however, counting the number of organoids after seeding. As we showed in Serra *et al.* (2019), the formation of organoids from single cells mimics a regenerative response (*3*). Therefore, what they show is that Myh-9 and Blebbistatin affect the number of organoids in a re-growth experiments after mechanical splitting (they call it number of survived crypts that might be a little misleading in this context). This increased stemness means that the organoids at the moment of crypt bulging and budding have more stem cells in the niche from the pre-treatment of blebbistatin in the first 36 hours. Then, they perform more experiments in which they look at number of crypts per organoids. However, as already mentioned the starting bulged organoid is different as they have been in Blebbistatin for full 36 hours. Moreover, the shape of their crypt is different than a control organoid and unfortunately they don't quantify it (wider crypt). This latter phenotype is very similar to ours when we add Blebbistatin on fully formed crypts (Fig. S5F). Another final aspect to consider is that Blebbistatin's lifetime in medium is between 10 and 12 hours (this is why we refresh medium more often in our experiments and we don't have information in the Zhao paper if they refresh medium in their 36 to 72 hours experiments). This means that the two set of experiments are not directly comparable as they look at a regenerative response mainly on organoid number without considering crypt morphogenesis while we focus on crypt morphogenesis and our perturbations are very specifically performed during a short window of time around crypt morphogenesis. To include this study of blebbistatin and Myh-9 in cell survival and regeneration, we added text in lines 183-184, page 5.

7. In Fig. S5C, the enrichment of villus basal Myh9 is not clear. Removing the signal from the dead cells may enhance the contrast.

We thank the reviewer for the suggestion. We tried manual and threshold-based methods of luminal signal removal, but it did not result in an improvement due to the low signal-to-noise ratio of the Myh-9-GFP intensity under time-lapse microscopy settings. We then tried different imaging processing strategies such as threshold-based method and deconvolution, and found that Huygens deconvolution was efficient at improving the signal-to-noise ratio of Myh9-GFP intensity. The newly processed movie now displays better the emergence of basal pool of Myh-9-GFP (Fig. S5D).

8. In Fig. S5D, n=2 is insufficient (line 1125).

New Fig. S5F increased the sample numbers in each group to Aphidicolin (n =22), Blebbistatin (n=13) with an additional control of DMSO (n=6).

9. In the caption of Fig. S5 (line 1129), it should be re-growth instead of re-grow.

Thank you for pointing this out. Due to change in the data, we changed the text accordingly.

10. Fig. S8D,E: How does the signal of Lgr5 change after inflation and collapse/deflation of the organoids?

To answer this, we now provide in our revised manuscript the data of the inflation experiments on organoid labelled by Lgr5-DTR-GFP. After inflation, we found that Lgr5-DTR-GFP expression does not show strong changes: the Lgr5 high cells before and after osmotic shock are the same, and the intensity of Lgr5 remains similar after osmotic shock. We have now added the new data to the manuscript (Fig. S9E).

11. In Fig. S8E, the authors aimed to show the effect of deflation using an osmotic shock on crypt emergence; however, the red arrows are not convincingly showing any emergence of crypts.

We thank the reviewer for raising this point. In the original submission, we applied osmotic shock on roundish organoids, which induces a bulging phenotype, although increasing the imaging resolution was indeed needed. In order to better show the emergence of crypts, we now updated these experiments by performing the same osmotic shock in Lgr5-DTR-GFP and H2B-iRFP labelled organoids, together with detailed 3D imaging. We believe that these new data show the phenotype in a much clearer manner and we have also updated the quantification accordingly (Fig. S9F).

12. In Fig. 4, the authors should explain how they have derived enterocysts. Moreover, it is not clear how the collapse of the inflated epithelium is related to the increase of enterocyte size. For example, luminal fluid could escape through the rupture of the epithelial monolayer or just go into a different part of the organoid, or even through paracellular leakage.

We thank the reviewer for this interesting point. Enterocysts, the organoids that consist of only enterocytes, are developed from the failure of symmetry breaking and Paneth-cell emergence, and are recognized by the failure of crypt budding as previously reported (*3*). We now explain it in the manuscript and add relative information in the method section.

To better address how does the tissue volume change coordinate with the lumen volume reduction, in the revised manuscript we increased sample number and calculated the change of the absolute tissue and lumen volume in each movie of enterocysts. As the reviewer pointed out, from our calculation, the tissue volume first increases and matches lumen volume reduction in a highly faithful manner (4 out of 5 measured enterocysts) (Reviewer Figure). Importantly, we had shown that this lumen volume reduction does not happens in organoids in presence of the Sglt1 inhibitor, providing an independent validation of the importance of osmotic effects for this relocation (Fig. 4 I-I'). However, in **one case** the tissue volumes stopped increasing and then reducing slightly while their lumen volumes kept reducing in the later stage of the movie (Reviewer Figure A). In such case not all of the luminal fluid goes into enterocytes but rather somewhere else.

Combining with what reviewer suggested, we would like to discuss other possibilities of where the not enterocyte-absorbed luminal fluid could go:

1) "luminal fluid could escape through the rupture of the epithelial monolayer". In healthy intestinal epithelium, the rupture of the epithelial monolayer or leakage is less likely to happen due to the critical barrier function of epithelium (*4*, *5*). One experimental evidence of absence of leakage in the intestinal organoid epithelium is that the intensity of autofluorescence in the organoid lumen rapidly increases during lumen volume reduction (supplementary movies 1, 2 and 8), supporting that the epithelium is intact enough to keep the autofluorescence signal inside.

   Moreover, in previous research studying luminal fluid escape that drives mouse blastocyst development, the lumen leakage is promoted by mitotic cell division (*6*). However, in the examples of enterocysts, which are composed of differentiated post-mitotic enterocytes, the lumen volumes are still significantly reduced, excluding the possibility of cell-division-driven tissue rupture.

   Finally, in CHIR-treated organoids (that lack enterocytes) the lumen volume increases without any leakage, supporting the role of enterocytes in absorbing the fluid.

2) "...or even through paracellular leakage". Paracellular water transfer does not cause increased enterocyte volume, and relies on blood flow *in vivo*, which is missing in the organoid culture. Moreover, *in vivo,* instead of paracellular water permeability, the transcellular pathway can transfer water against luminal hypertonic condition through the regulation of cellular osmotic gradient (*7*). In our movies, tissue volumes increase with lumen volumes reduction in the first period, indicating primarily the absorption activity in enterocytes drives lumen shrinkage. If absorbed water reaches the limit of enterocyte volume, the transcellular rather than paracellular pathway can further facilitate water transfer, resulting in constant (or even slightly reduced) tissue volume and reduced lumen volume in the later stage in the one enterocyst movie (Reviewer Figure A) that behaves differently than the others (Reviewer Figure B-E). In fact, in this specific sample, the reduction of lumen volume is higher than the rest of samples, supporting the possibility of exceeding the capacity of enterocyte absorption. Last but not least, perturbations on transcellular pathway through the inhibition of the AQPs and Na^+/K^+ ATPase led to mild lumen shrinkage defect (Fig. S10E), indicating that transcellular water transfer could guide the relocation of luminal liquid.

Taken together, we agree with the reviewer and in the current version of the manuscript we explained better "how the collapse of the inflated epithelium is related to the increase of enterocyte size" and discuss the possibility of transcellular water transfer. Therefore, we re-plotted Fig. 4 with 5 enterocyst movies and added explanation for the enterocyte movies in method section.

13. Line 305 of the main text refers to Fig. S9D and not Fig. S9C.
14. Line 314 of the main text refers to Fig. 4H.
15. Moreover, line 316 and 318 refer to Fig. 4I-I' and S9E instead of Fig.4H and Fig.S9D.

We thank the reviewer for noticing these typos and have corrected them.

16. How do the ion channel piezo1 or piezo2 as mechanosensors affect the emergence of bulge or bud in the organoids? Experiments should be conducted using an activator (Yoda-1) or inhibitor (GdCl3) of these piezo channels.

We thank reviewer for the question and suggestion.

We checked the expression of *piezo1* and *piezo2* in single-cell RNAseq (scRNAseq) data. The expression of *piezo1* is rare and randomly distributed in cells from both crypt and villus, while *piezo2* was not detected. We further analysed the expression of Piezo proteins by immunostaining with several Piezo1 and Piezo2 antibodies. Neither Piezo1 or Piezo2 show tissue specific enrichment, nor high expression. Piezo1 occasionally exhibits weak expression in a few single cells, which matches the detection of mRNA in scRNAseq data (Fig. S11A and B). Due to the low and unspecific tissue expression of Piezo channels, we had not added them as candidates to the list of ion channels for further functional analysis in previous manuscript.

Activation of Piezo1 is known to induce cations entry into cell (*8*), which could increase cellular osmolarity and reduce lumen volume during crypt budding. Therefore, we tested, as reviewer1 suggested, the activator (Yoda-1) and inhibitor (GdCl3) of Piezo1, in addition to another inhibitor (spider venom peptide, GsMtx4), and performed time-course experiments of organoid development and crypt formation.

As expected from the low Piezo expression, inhibiting Piezo channels by GdCl3 and GsMtx4 did not show significant defect in lumen shrinkage and crypt budding (Fig. S11C-E). This is in contrast to our previous data on the inhibition of other ion channels specifically enriched in enterocytes (Sglt-1) or highly expressed in the whole epithelium (Aquaporins and Na^{+}/K^{+} ATPase), which had stronger effects on lumen shrinkage/crypt budding. These results confirm that Piezo1 is not a strong regulator of crypt morphogenesis in the similar way as Sglt-1, Aquaporins and Na^{+}/K^{+} ATPase.

However, activating Piezo1 by Yoda-1 did cause slightly increased lumen volume and reduced eccentricity (Fig. S11C -E). Previous study of stretch-activated Piezo channel in Drosophila mid-gut has demonstrated the function of Piezo in promoting stem cell differentiation towards the enteroendocrine lineage (*9*). Thus, the reduced lumen shrinkage in Yoda-1-treated organoids could be a consequence of the reduced number of absorptive enterocytes that up-taken the lumen volume. Indeed, from the detection of enterocyte fate in Yoda-1-treated samples, we observed the reduced Aldolase B staining of enterocytes in the villus region (Fig. S11C).
The data have been added to the manuscript in Fig. S11, text lines 357-366 in page 9.

Reviewer #2
Remarks to the Author:
Yang and Xue and colleagues follow up on previous work from the Liberali lab that showed how intestinal organoids/enteroids break symmetry after initially forming as a uniform cystic structure. In the current work, the authors focus on the cellular mechanisms that drive the process of bulging and budding during crypt formation in organoids following this symmetry breaking event. They demonstrate that apical contraction in crypts and basal tension in the villus generate curvatures leading to crypt formation, and that enterocytes contribute to this process by swelling.

Overall this is a strong manuscript. It validates qualitative observations with rigorous quantitative analysis, and also provides mathematical modeling and theory to describe the observations being made. Thus, claims made are mostly supported with compelling data.
We thank the reviewer for his support and constructive comments, which we address below.

I had only a handful of comments/critiques/questions:

Major points:

Is the differentiation of a Paneth cell at all correlated with apical constriction and crypt formation? Would crypts/buds form in the absence of PCs, such as in the ATOH1-null epithelium? Certainly crypts seem to form just fine in genetic ATOH1-null animals; however since this group has attributed the symmetry breaking (and initiation of bulging/budding) of the cyst in part to PCs, it would be interesting to know if PC are in any way correlated to Myh-9-GFP in the context of organoid crypt budding.
We thank reviewer for the interesting questions. We do not have access to the ATOH1-null animals, and we used other experimental procedures to have organoids with reduced Paneth Cell. Therefore,

to address this question, we performed new experiments comparing Myh-9-GFP in enterocyst, in organoids enriched with Paneth cell (PC, treated CHIR+DAPT), or stem cell (SC, treated CHIR+VPA) (here for simplification, we term the different organoid types as PC organoid and SC organoid) (Fig. S6). Comparing to enterocysts that have more basal Myh-9-GFP (matching villus tissue in organoids), the PC organoid and SC organoids all have higher apical Myh-9-GFP signal (Fig. S6C, C' and E). Interestingly, SC organoids have the highest apical *vs.* basal Myh-9-GFP ratio, arguing that stem cells have the highest contribution to spontaneous curvature. Even in the PC organoid, very few Lgr5$^+$ stem cells can still remain, and we find that these generate higher regional spontaneous curvature leading to slight local bulges (Fig. S6A, red arrows). Altogether, these data suggest that stem cells in crypt tissue are the dominant force creating the actomyosin based apical constriction necessary for spontaneous curvature, although Paneth cells could of course have a smaller, but non-zero contribution. We now discuss this in lines 201-214, pages 5-6 of the main text and added these data in Fig. S6.

As a mechanism, the authors explore differential localization of Z01, Occludin, Claudin2 and N-cadherin. They observe interesting crypt-specific patterns of Occludin, Claudin2 and N-cadherin while Z01 was expressed in the crypt and villus apical surface and in the villus basal surface. They conclude "However, among them, only ZO-1 overlaps with the basal pool of Myh-9-GFP in organoid villus region, and exhibits villus basolateral localization both in vitro and in vivo during crypt morphogenesis". Yet, the authors do not provide in vivo data for Occludin, Claudin2 or N-cadherin. Moreover, they did not look at any of the markers with high temporal resolution around the time of crypt emergence in vivo (i.e. around Post-natal day 14). It is possible that both basal Z01 in the villus region coupled with apical Occludin/Claudin2/N-cadherin in the crypt domain are both important for the process of actomyosin patterning and crypt budding. It seems that the conclusion that Z01 contributes to the emergence of the region-specific actomyosin pattern overlooks this possibility. Some additional immunostaining in vivo during the time of crypt formation will be helpful to add this valuable information.

We thank the reviewer for the suggestion, we now tested multiple antibodies against tight junctions in a new time-course of crypt formation *in vivo*. In the revised manuscript, we present Claudin-2 and ZO-1 *in vivo* staining at P1, P2, P5, P7, P11, P12, P13, P14, P15, P16, P17 and 6-month-adult stages (Fig. S6F and G). Unfortunately, Occludin and N-cadherin were not successfully stained in *in vivo* tissue. The data show that Claudin-2 exhibits high apical and weak basolateral localization in the gradually matured crypt regions. ZO-1 localization was at the apical junction through the whole epithelium, and enriched at basolateral side in gradually matured villus tissues during development (Fig. S7F and G). Importantly, these *in vivo* localization patterns of both Claudin-2 and ZO-1 matched well to their patterns in organoids (Fig. S7E-G), which showed high Claudin-2 at the crypt apical side together with high ZO-1 at apical junctions and villus basolateral side.

We, however, agree with the reviewer that the dual-localization of ZO-1 on the apical side and basolateral does not mean that only ZO-1 is important for crypt budding and that it is possible that basal ZO-1 in the villus region coupled with apical Occludin/Claudin-2/N-cadherin in the crypt domain are both important for the process of actomyosin patterning and crypt budding. We therefore changed the text in lines 223-231, page 6.

For lumen volume experiments that used osmotic shock – the authors should show that cells are viable and that the cells themselves are not affected by the osmotic shock. Should the osmotic shock affect the cells themselves (and not just lumen volume), the current interpretation of the experiment would likely need to be revised.

To demonstrate better the change of individual cells with lumen volume reduction, we integrated the construct of H2B-iRFP reporter into the genome of Lgr5-DTR-GFP organoid and applied osmotic shock on this Lgr5-DTR-GFP, H2B-iRFP dual-reporter labelled organoid line, and recorded with 3D spinning disk confocal imaging. Our result demonstrates that before and after osmotic shock, cells were not strongly affected. Yet the Day 3 DMSO-treated roundish organoids are able to bulge, and the Day 3.5 DMSO-treated bulged organoids are able to bud (Fig. S9F).

Also – for these experiments, the authors concluded "seconds after the osmotic deflation, the Day3.5 organoids formed bulges in the crypt regions enriched in Lgr-5+ stem cells, while the Day2.5 and Day3.5 CHIR-treated organoids remained spherical and did not display significant bulging"; however, in the eccentricity measurement, the day 3.5 CHIR showed statistically significant increased eccentricity after osmotic shock (Fig 8SE – box plot). Is this conclusion at odds with the statistical analysis?
We thank the referee for noticing this. Going back to the data, we found that this slightly increased eccentricity was because Day 3.5 CHIR treated organoids were already not fully homogeneous as Day 2.5 cysts. Although less differentiated compared to Day 3.5 organoids, some Day 3.5 CHIR-treated organoids could still have clustered $Lgr5^{+}$ stem cells and Paneth cells. Therefore, upon osmotic shock, Day 3.5 CHIR treated organoids could still bulge a bit and have slightly higher eccentricity than Day 2.5 organoids due to slight differential tissue spontaneous curvature.

To avoid using partially differentiated Day 3.5 CHIR-treated organoids, we now selected in the revised manuscript a more homogenous population of Day 3.5 CHIR-treated organoids (based on their roundish organoid shape and evenly distributed Lgr-5-DTR-GFP). Osmotic shock on these homogenous Day 3.5 CHIR-treated organoids demonstrated no significant change on eccentricity (Fig. S9F).

Figure 4D – are the authors claiming that total enterocyst volume does not change? It seems that lumen volume goes down, cell volume does go up, but total volume goes up initially and then back down. Can the total loss of lumen volume be accounted for by the increase in cell volume? It does not seem like cell volume increases sufficiently to account for 100% of lumen volume loss, but this is something the authors could calculate.
We thank the reviewer for the questions.
To better address how does the cell (tissue) volume change coordinate with the lumen volume reduction, in the revised manuscript we increased sample number and calculated the change of the absolute tissue and lumen volume in each movie of enterocysts. As the reviewer pointed out, from our calculation, the tissue volume first increases and correlates with lumen volume reduction (total volume therefore remains or increases slightly) in a highly faithful manner (4 out of 5 measured enterocysts) (Reviewer Figure). However, in one case the tissue volumes stopped increasing and then reducing slightly while the lumen volumes kept reducing in the later stage of the movies (Reviewer Figure A). In such case the cell volume does not increase sufficiently to account for 100% of lumen volume loss. However, on average the total volume is constant with up to 7% variation.

In the current version of the manuscript, we re-plotted Fig. 4D with 5 time-lapse recordings and added explanation of the enterocyte movies in method section.

Minor points:

Perhaps semantics or differences of word usage in different fields (i.e. biological vs. mathematical/theoretical), but the authors refer to the change in curvatures that lead to crypt formation as "spontaneous"; however, their previous work (Serra and Mayr et al.) demonstrated that the process of symmetry breaking is very stereotyped and reproducible. That is, the formation/differentiation of a Paneth cell preceeds budding. Thus, the word "spontaneous" seems rather at odds with an active and stereotyped process in the biological sense.
We thank the referee for prompting this clarification. We use the word "spontaneous" here in analogy to the lipid membrane literature, where it doesn't refer to symmetry-breaking, but is rather

synonymous to "intrinsic", to say that cells with apical myosin, like lipid with different head-tail sizes, have a preferred curved shape.

We have now added a clarification when we first introduce this term, to clarify this synonymous to "intrinsic", i.e. the preferred shape (at mechanical equilibrium) of a given stem cell being to be curved (lines 120-123, page 3).

The authors nicely show using LifeAct and Myh-9-GFP, coupled with direct measurements of the tension in the basal surface of crypts vs. villi that there are differential tensions and localization of Myh-9-GFP on the basal surface. Assuming that the epithelium of both the crypt and villus region are interacting with the homogeneous extracellular matrix (Matrigel) during the process of crypt budding, have the authors examined the interaction of the crypt and villus epithelium with the surrounding matrix, and is there any evidence that this interaction (i.e. remodeling of matrix proteins) is correlated with the crypt region vs. the villus region? That is, is the matrix also an active participant in this process, or a passive bystander that simply gets pushed around by epithelial forces?

We thank the reviewer for the question. To examine the effect of extracellular matrix (ECM)/ remodelling of ECM on crypt budding, we have now performed drug treatment with two broad spectrum inhibitors of matrix metalloproteinases (MMPs) (GM6001 and Marimastat).

Treatment of both MMP inhibitors show that formation and morphologies of crypts were not significantly different between the MMPi-treated and the DMSO-treated organoids (Fig. S12), indicating the remodelling of ECM is not required for organoid crypt budding. Moreover, when we prepared microinjection and micropipette aspiration experiments, removing Matrigel from budded organoids did not reverse the crypt budding morphology. Taken together, we conclude that in the scenario of crypt morphogenesis, the matrix does not have a strong mechanical contribution to crypt budding.

In Figure 2B, it will be helpful if the authors label each of the 3 scenarios in the schematic corresponding to their numerical assignments in the text (i.e. scenario "i)", "ii)", and "iii)").

We have followed the advice and changed in Fig. 2B and figure legend.

In the text the authors state: "This provides a key qualitative test of the mechanism we propose: a mechanism of softer crypts would result in the reverse trend of preferential crypt expansion, a mechanism of budding via crypt cell proliferation (i.e. buckling) would result in crypt opening as fluid injection increases the area/volume ratio (22) (see SI Text for detailed discussion)." Here, the authors state two alternative possibilities to the mechanism they propose. It will be helpful for the readers if they re-state their proposed mechanism first, and explicitly call the discussion points above "alternative mechanisms", which do not fit their model.

We followed the advice in the revised text (lines 272-278, page 7) to more clearly state each scenario sequentially, and remind the readers of the one we propose (spontaneous curvature).

For Figure 4A – were enterocysts and CHIR grown organoids given osmotic shock? The section heading indicates this is the case, but the text and figure legend do not explicitly state they were treated with osmotic shock.

The enterocysts and CHIR grown organoids were not given osmotic shock.

Fig. 4A indicates the phenotypes of enterocysts and CHIR-treated organoid in Fig. 4B and C, which are the growth of enterocysts and CHIR-treated organoids without osmotic shock. These two experiments reveal enterocytes is the cell type responsible for lumen shrinkage.

The "Osmotic changes" in the section heading describes the membrane transporters-driven osmotic changes in enterocyte that can lead to luminal water relocation.

Figure 4 panels are mis-labeled in the text (i.e. figure 4G).

We corrected them in the revised text.

Reviewer #3

Remarks to the Author:

Summary:

In this study, the authors use a 3D organoid culture model to identify a mechanical mechanism for crypt morphogenesis. The authors examine the effects of actomyosin-driven apical contraction, villus basal tension, lumen volume and tissue volume on the geometry of organoids with developing intestinal crypts both experimentally and in a biophysical model. The authors nicely use their model to guide experimental perturbations that are then used to confirm model predictions and find that differential spontaneous curvature in the crypt vs. villus region together with lumen volume reduction can explain the morphological changes during crypt budding. The differential curvature nicely matches the pattern of myosin localization at the apical side of the bulging crypt and at the basal side of the villus region leading to increased tension as demonstrated by laser nanosurgery and micropipette aspiration assays. Upon inflation of the lumen using pharmaceutical and mechanical methods, budded crypts could not be opened, but less developed bulged crypts could be as predicted by the model. Furthermore, lumen volume was found to be osmotically redistributed from the lumen to the enterocytes in the villus region, increasing compressive stress on the crypt and thereby supporting budding. The authors demonstrated that although the geometry in vivo has an open lumen, swelling of cells in the villus could still contribute to crypt morphogenesis in vivo. In conclusion, the authors elegantly overcome the difficulty of studying an internal organ with limited accessibility by taking advantage of live imaging of intestinal organoids in combination with biophysical modeling to study the mechanical mechanism of crypt formation.

Major Points:

• The title of the paper comprises the term “cell fate coordinates”, however how cell fate leads to differential apical actin constriction in the crypt region is not a major focus of the paper. I suggest to either include more data on how cell fate controls apical constriction and thereby tissue curvature or rephrase the title to better represent the focus of the paper on apical/basal tension, tissue curvature, lumen volume and cell swelling.

We thank reviewer for the interesting question. To address this question, we preformed new experiments comparing Myh-9-GFP in enterocyst, in organoids enriched with Paneth cell (PC, treated CHIR+DAPT), or stem cell (SC, treated CHIR+VPA) (here for simplification, we term the different organoid types as PC organoid and SC organoid) (Fig. S6).

Comparing to enterocysts that have more basal Myh-9-GFP (matching villus tissue in budded organoids), the PC organoid and SC organoids all have higher apical Myh-9-GFP signal (Fig. S12E and G). Interestingly, SC organoids have the highest apical *vs.* basal Myh-9-GFP ratio, arguing that stem cells have the highest contribution to spontaneous curvature. Even in the PC organoid, very few $Lgr5^+$ stem cells can still remain, and we find that these generate higher regional spontaneous curvature leading to slight local bulges (Fig. S6A, red arrows). Altogether, these data suggest that stem cells in crypt tissue are the dominant force creating the actomyosin based apical constriction necessary for apical constriction, although Paneth cells could of course have a smaller, but non-zero contribution. We now discuss this in lines 201-214, pages 5-6 of the main text on stem cell fate controlling apical constriction, and added these data in Fig. S6.

• Lumen shrinkage and epithelial volume increase are nicely demonstrated. However, the authors do not discuss the possibility of local differences in cell division contributing to the crypt vs. villus epithelium differentially. Are there local differences in cell division and if yes, how would they affect the model?

We thank reviewer for prompting us to clarify this question, which we realized was unclear in our original submission. Indeed, there are local difference in cell division, with divisions occurring predominantly in the crypt regions (as *in vivo*). To test this role, we had used Aphidicolin treatment to block mitotic cell division in budded organoids (Fig. S5F). Importantly, their morphology displayed no difference with the DMSO-treated organoids. Moreover, we had also used Blebbistatin treatment to disrupt contractility, which in contrast significantly disrupt crypt budding. As we make clearer in this revised version, this argues against local differences in cell division creating residual stresses in the crypt causing it to bulge (as such "division-induced buckling" mechanism should then not be reversed by contractility). However, division is indeed indirectly taken into account in our model – which is quasi-static given the timescales – through the geometric parameter \phi (the relative size of the crypt domain). This parameter, which we measure independently when fitting the inflation or morphogenetic evolution of organoids (Fig. 2 and 3), increases between bulged and budded organoids due to divisions in crypts and thus takes into account the difference in division in the model. We clarified this by detailing more the theoretical model in main text (lines 186 -187, page 5 and lines 275-278, page 7) and in Supplementary (lines 598 - 627, Section 1.5, pages 19-20).

• The authors note that the mesenchyme is important for the formation of villi in vivo and say that the organoids can form a crypt and villus without mesenchyme. Could the authors discuss possible effects of the mesenchyme on crypt morphogenesis in vivo, especially how it would impact the mechanical landscape? This would be helpful and contextualize the work better in existing work on intestinal development (e.g. references 5, 11).

This is an interesting point. Whereas crypts are extremely similar between organoid and *in vivo* intestine (both in cellular composition and overall shape/morphology) (*10*), it's interesting that distinct villus shapes are absent in organoids. As villus morphogenesis has been proposed to be dependent on buckling from the mesenchyme/smooth muscle(*11*), while crypt morphogenesis occurs much later in development, it is tempting to speculate that mesenchyme could play a stronger role in the morphogenesis of the former rather than the latter. We comment on this in the discussion of the revised manuscript (lines 408-413, page 10).

Minor Points:

• The term "spontaneous curvature" is central to the manuscript and should be explained to readers who are not familiar with this terminology. The term "spontaneous curvature" should be clearly separated from the term "spontaneous symmetry breaking" to prevent misunderstandings. If I understand correctly, cell fate leads to local differences in tissue curvature and crypts do not form because of a spontaneous local increase in curvature at a random position of the spheroid.

The referee is perfectly correct in his interpretation – we use the word "spontaneous" here in analogy to the lipid membrane literature, but now added a clarification when we first introduce this term that it means rather because of an "intrinsic" curvature that is acquired by stem cells (lines 120-123, page 3).

• One of the major points in the paper is that the spontaneous curvature of the tissue drives morphogenesis. However, the curvature of the villus epithelium in the organoids is inverted compared to the in vivo situation. Could the authors comment/discuss this point?

As discussed above, given the reports of villus morphogenesis being dependent on mesenchyme/smooth muscle induced buckling, we believe this is why the villus does not show reverse curvature (for large organoids the radius of curvature of villus cells will tend to zero, which is the state of the pre-buckled intestine *in vivo*). We clarified this together with the previous discussion in lines 406-413, page 10.

• The authors propose that osmotic transport of fluid into the enterocytes in the villus results in lumen volume reduction. In Supplementary Figure 9, they track crypt and villus cell volume changes. A figure showing the volume reduction in the lumen side by side with the total volume increase in the villus epithelium (or a similar comparison) would strengthen this point and add additional quantification to it.

We thank the referee for this helpful suggestion and have now provided organoid lumen ratio with single-cell volume change in Fig. S10B, and correspondingly put in Fig. S10C the *in vivo* re-measured distance between villi based on the same marker staining (Beta-catenin).

• Ref. 11 talks about a "hinge" between the villus and crypt. When the authors discuss in vivo applications of their findings, incorporating a short comment on effects the hinge region would have on morphogenesis would be helpful.

Reference 11 shows at the developmental stage of P20, Rac1 prevents integrin-guided hemidesmosomal attachment of epithelium to ECM, which allows cell basal constriction at the crypt border, resulting in proper villar spacing after crypt budding (*12*). However, Rac1 loss-of-function at earlier stages did not cause any defect in crypt morphogenesis. We therefore think that hinge cells are likely to be important to stabilize crypt shape at later stages than the ones we consider here. This could in principle be modelled by the "boundary" term that we had included in our energy to represent possible specific mechanical contributions of the crypt-villus boundary. We now discuss this in lines 392-396, page 10 of the revised discussion.

• Figure 2D: The figure panels are very small. Please increase the size by using empty white space in the figure to allow the reader to see the nanosurgery experiments.
• Figure 2D'': The error bars overlap and are not easy to read. Incorporating a design similar to Fig. 1E would improve readability.
• Figure 2E: Include a panel showing a zoom-in onto the micropipette so that the reader can see the difference in basal tension in the crypt vs vilus region.

We have followed the advices.

• Figure 2F, F', F'', F''': The authors show an image of basal Myh-9-GFP intensity in the villus and crypt, but do not quantify or directly compare the two, instead incorporating them into an overall intensity in F''. A figure similar to F' but for the basal side would be helpful to compare myosin between the two regions on the basal side (presumably important for maintaining villus tension).

We thank the referee for his careful reading of the figures. We quantified the basal Myh-9-GFP in different stages and integrated the plot into Fig. 2G.

• Figure 2 legend: The legend is very long. Consider shortening for example by moving information about method details (for the micropipette aspiration assay) to the method section rather than the figure legend.

We have followed the advice.

• Methods section: please make sure to explain abbreviations used such as FCN
• Typos in the text:
o Line 61: "Day3" and "Day4" are missing blanks
o Line 137: There is no Fig. 2C'''. Please refer to the correct figure panel.
o Line 151: Fig. 2D-D'' should read Fig. 2D-D'''.
o Line 314: "Fig. 4G" should be replaced with "Fig. 4H".
o Line 318: "Fig. 4H" should be replaced with "Fig. 4I".

We thank the referee for pointing these and have corrected these typos and abbreviation definitions.

Reference:

1. M. D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, A global double-fluorescent Cre reporter mouse. *genesis*. **45**, 593–605 (2007).

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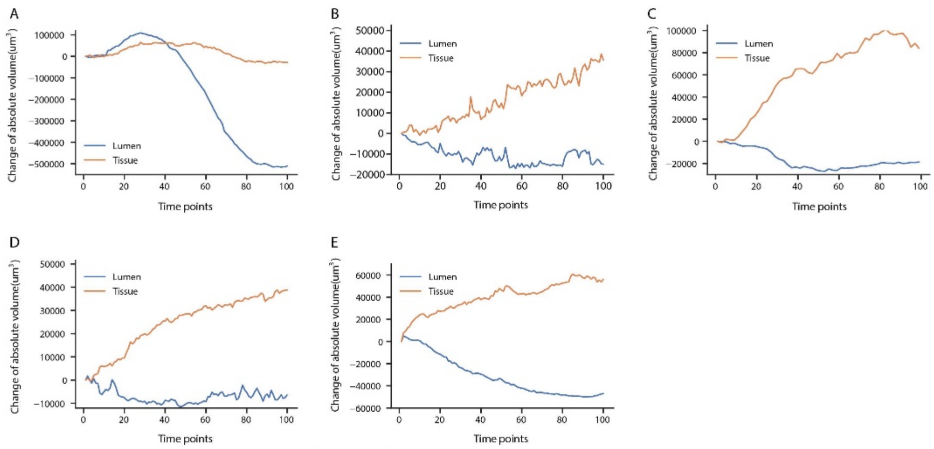
12. K. D. Sumigray, M. Terwilliger, T. Lechler, Morphogenesis and Compartmentalization of the Intestinal Crypt. *Dev. Cell*. **45**, 183–197.e5 (2018).



Reviewer Figure. Change of the absolute lumen and tissue volume in development of enterocysts. A – E plot the volume dynamics of each individual enterocyst.

Decision Letter, first revision:

Date: 18th March 21 07:32:57
Last Sent: 18th March 21 07:32:57
Triggered By: Christine Weber
From: christine.weber@nature.com
To: prisca.liberali@fmi.ch
CC: ncb@springernature.com
Subject: Your manuscript, NCB-L43714A
Message: Dear Prisca,

Thank you for submitting your revised manuscript "Cell fate coordinates mechano-osmotic forces in intestinal crypt formation" (NCB-L43714A) to Nature Cell Biology.

It has now been seen by the original referees and their comments are below. As you will see, the reviewers find that the paper has been improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy referee 1's final request and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in 1-2 weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

As the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex) -- we cannot proceed with PDFs at this stage.

Thank you again for your interest in Nature Cell Biology. Please do not hesitate to contact me if you have any questions.

With best wishes,

Christine.

Christine Weber, PhD
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Reviewer #1 (Remarks to the Author):

The authors have done a nice job in addressing my concerns. The only minor comment is that the typo introduced in the conversion of the equations (line 362 and 379 of the Supplementary information) has not been corrected yet.

Reviewer #2 (Remarks to the Author):

The authors have nicely addressed every criticism from this reviewer. An already strong manuscript is now even better, and I believe that it is suitable for publication in Nature Cell Biology.

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed the reviewer concerns. The revised manuscript provides an excellent description of the mechanisms by which crypts form in intestinal organoids.

Author Rebuttal, first revision:

Reviewer #1:
Remarks to the Author:
The authors have done a nice job in addressing my concerns. The only minor comment is that the typo introduced in the conversion of the equations (line 362 and 379 of the Supplementary information) has not been corrected yet.

We thank the referee for pointing the typo and have corrected it for equation 6 (line 85) and equation 7 (line 102) in the current version of the supplementary note file.

Final Decision Letter:

Dear Prisca,

I am pleased to inform you that your manuscript, "Cell fate coordinates mechano-osmotic forces in intestinal crypt formation", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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