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Cell influx and contractile actomyosin force drive mammary bud growth and invagination

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October 5, 2020

Re: JCB manuscript #202008062

Dr. Marja L Yam
Institute of Biotechnology
P.O.Box 56
University of Helsinki 00014
Finland

Dear Dr. Mikkola,

Thank you for submitting your manuscript entitled "Cell hypertrophy, influx and contractile actomyosin force drive mammary bud growth and invagination". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can directly resolve the key concerns of the three conscientious and highly expert reviewers.

A revised manuscript would need to resolve as directly as possible the constructive concerns of these referees by more precise wording and ideally better and clearer evidence. Morphometric quantification would be quite helpful, and precise definitions that other researchers can use to identify ring cells when repeating or extending this research will be important. If practical, evaluation of later development would be helpful. Please make every effort to resolve or clarify the other specific points raised by these very perceptive peer reviewers, though we feel that the decision about where to show figures should be yours after considering the comments.

If you can provide a resubmitted manuscript that resolves the substantive concerns of these expert reviewers, which we hope will be possible, it will be returned for final re-reviewing to Reviewer 1 and Reviewer 3 to determine whether the concerns have been sufficiently resolved.

Thank you for submitting this intriguing paper to JCB.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Kenneth Yamada, MD, PhD
Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

General comments:
This manuscript uses confocal microscopy analysis of whole-mount embryonic mouse mammary glands to study the growth and invagination of the mammary bud. The authors report that placode formation is largely driven by cell migration. They also report that epidermal cells with high levels of contractility called ring cells are important for invagination and the formation of the mammary bud neck. Using a Myh9 conditional knock-out to compromise the contractility of ring cells, they find that both invagination and neck formation are impaired. In general, the reviewer finds that both the design and execution of this study are communicated clearly. Once the recommended revisions are

complete, the reviewer believes that this manuscript will be suitable for publication in the Journal of Cell Biology.

Specific concerns:
1. Page 7: The statement "these results indicate that during early mammogenesis the gland slowly grows and rapidly changes its shape..." do not seem consistent with the data presented in Figure 1D and E. Figure 1D shows that the volume significantly increases from E11.5 to E12.5 and from E12.5 to E13.5, and Figure 1E shows that sphericity only increases significantly from E11.5 to E12.5. Given these results, how can the growth be described as slow and the change in geometry be described as fast? Significant changes in sphericity and volume are observed over the same development time period (E11.5 to E12.5).
2. Page 10: The statement "We considered that cells presumed to migrate towards the mammary gland placode would have an angle of less than 90 degrees..." is confusing. How was this criterion established? Couldn't cells with an angle of less than 90 degrees migrate away or tangential to the placode?
3. Figure 3E and F are difficult to interpret. Firstly, the white arrows used to denote the polarity of cells are hard to read. It would be helpful if these were replaced with the vector format that is used in supplementary figure 3C. Also, the results in Figure 3F would be easier to interpret if they were plotted using the same format as the rose plot shown in Figure 4G. Lastly, the conclusion that cells are migrating into the placode would be best supported by cell trajectories obtained using live imaging, although this may not be feasible. If live-imaging is not feasible, it may be useful to reanalyze existing data to observe any migration occurring in the Z direction.
4. Page 10: The phrase "these cells showed a honeycomb-like alignment" is confusing. Doesn't the honeycomb pattern result from orientation of the cell borders/shape of cells? The statement would be easier to interpret if it stated that cell-cell borders formed a honeycomb pattern, which indicated that cells had a hexagonal shape.
5. Page 11: The authors term the cells surrounding the mammary hillock "ring cells" due to "their particular appearance and arrangement." A more quantitative description of these cells here could be useful for the rest of the paper. For example, does the term ring cell refer to specific cell aspect ratio? Or a distance out from the hillock? Later, when the authors describe the cells disappearing, it would be useful to be able to refer to a specific quantitative reason why cells are no longer termed ring cells.
6. Figure 4: The plots in Figure 4D have a bar that represents ring cells at stage E13.5 even though the authors acknowledge that "At E13.5, when ring cells had disappeared...". Are the "ring cells" at E13.5 in these plots actually the "neck cells" that the authors refer to in the corresponding text?
7. The authors make two claims about the Myh9 cKO condition: "Ring cells...were less pronounced than controls..." and "reduced F-actin and pMLC levels at all stages analyzed (Fig. 6A-C, S5 A-C.)". Quantification is needed to confirm these conclusions. Specifically, a metric is needed for identifying how pronounced the ring cells are. Maybe the Feret diameter or aspect ratio would be helpful for this analysis. Also, a plot comparing the intensity of F-actin and pMLC in the control and cKO condition is required.
8. The results in Figure 7B and the statement "One day later, after invagination had occurred, the protrusion was no longer evident (Fig. 7 B)" are confusing. Adding a schematic that clearly emphasizes the tissue layers that are observed in the SEM data as compared to the immunofluorescence staining would be helpful.
9. The authors reference Figure 7G in the main text, but Figure 7G does not exist. Do they mean Figure 7F?
10. Related to a previous comment, quantification is needed to support the statement "ring cells become further pronounced (Fig. 7 B)"
11. In the discussion, the authors state that "Importantly, their morphology and contractility were

severely impaired upon conditional deletion of NMIIA..." Quantification of the morphology of keratinocytes in both the control and the cKO condition is required to support this claim. Otherwise, the authors are overstating the findings of this study.

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

This paper addresses the cellular mechanisms involved in mammary placode formation and bud invagination. The authors have used state of the art techniques (including 3D confocal microscopy, quantitative image analysis and 3D surface rendering, and extensive use of genetically modified mouse mutants to facilitate imaging and study nuclear shape, cell cycle dynamics etc.) to bear upon the basic question of appendage morphogenesis. The data presented are of exceptionally high quality, the imaging is beautiful and the quantitation is exemplary throughout.

The authors have previously studied mechanisms governing tooth and hair follicle invagination. This report now adds important new information on mammary primordia and will be of interest to all studying epidermal appendage development.

Using cutting edge techniques, they exclude cell proliferation in placode development and show hypertrophy plays a minor role promoting initial placode formation. Instead, they identify cell migration as the critical mechanism. Previous studies have shown lack of cell proliferation, and suggested hypertrophy and cell migration are responsible - but none have approached the quality of the analyses contained in this study, which now provides definitive evidence.

The authors go onto show that cell influx leads to the next stage of hillock formation. The major finding of the paper is the introduction of the concept of a contractile circumferential ring of elongated epidermal cells surrounding the primordium that assists in propelling invagination. A role for surrounding epidermis in this process is entirely novel and this cellular rearrangement has not been noticed before and is intriguing. The authors make a thorough description of this novel structure and then demonstrate genetically, by conditional deletion of non-muscle myosin IIA, that contraction of "ring cells" is required to form the characteristic neck region of the flask shaped mammary bud. While invagination still occurs (likely due to redundancy issues) it is nevertheless delayed and impaired and the constriction of the neck region is lost altogether.

In my opinion the authors have made an important contribution to our understanding of the cell biological mechanisms of morphogenesis of stratified tissues. They present an interesting discussion comparing the distinct mechanisms employed by different appendages during the invagination process.

I have only minor criticisms and suggestions as follows that mostly concern the writing

1. Some headings and statements (e.g. The first subtitle "reveals a role for cellular hypertrophy") are a bit misleading as they give the impression that hypertrophy is a major mechanism whereas the data and final conclusion of this section of the results show it is minor. The authors should present this finding more confidently. Suggest removing the term hypertrophy from the title and adjusting the subheading to be consistent with the data and conclusion.

2. Figure 7 is the most important part of the paper but the writing in the Results section is extremely hard to follow - in its current form it is unclear where the control or the mutant is being

described. Lines 303-309 definitely need rewriting.

3. The figure legends are very detailed but give little guidance as to the main points the reader is supposed to look at in the images - in several arrows are in the figures but there is no explanation in the legend as to what they are indicating.

4. The discussion would benefit from being shortened a little. For example, suggest cutting out lines 344-351

5. The term ring cells doesn't convey the supracellular nature of this structure or its proposed mechanism- maybe something like - epidermal contractile ring?? (Just a suggestion).

6. Minor points:

a. Line 88 "The First...." Make word "first" lowercase
b. Line 132 "enlarged" - this term is too vague and does not apply to the first part of figure which shows reduced surface area - suggest use "enlarged volume" and refer specifically to the reconstruction part of the figure
c. Line 400 "describe and undescribed" needs rephrasing
d. Line 855 (Fig 3 legend) - need to reverse the order of Epcam and Hoechst in sentence: of "Cell nuclei and epithelial cells are stained with EpCAM (white) and Hoechst (cyan), respectively.
e. Fig 5C and throughout the text don't use "expression" - it's just levels by immunofluorescence- perhaps "intensity" is safer as relocalization rather than upregulated gene expression seems more likely to account for it.
f. Throughout text check if use of mammary gland is appropriate - in some cases just "mammary" is sufficient e.g. mammary fate and in others perhaps "primordium" or "rudiment" might be better than "gland" as its glandular nature is not yet acquired at E12-15.

Reviewer #3 (Comments to the Authors (Required)):

In the present manuscript, Trela and colleagues analyze the earliest steps of mammary gland morphogenesis. They address several interesting and unexplored questions about the mechanisms leading to placode invagination and neck formation, two processes necessary for the successive branching development of the mammary epithelium. This aspect of primitive mammary development has not been thoroughly investigated and for this reason the paper is rather exciting. The authors perform accurate measurements of mammary bud growth as well as cell numbers, behavior and polarity. However, the presented data is for the most part simply descriptive and I was left with the impression that the authors draw speculative conclusions by overinterpreting their results. Even when they use a cKO mouse, the subtle phenotype observed does not provide sufficient and compelling evidence for an essential role for MyosinIIA in ring cells.

I will list here the data that are in my view problematic because of the strong conclusions drawn in the manuscript:

1- The authors study the dynamics of volume gain during mammary growth from placode to bulb. Based on the data presented in Fig. 2, they claim having "compelling evidence" for a "proliferation-independent" growth mechanism. I disagree with this conclusion as in Fig. 2G we can appreciate that 6-9% of the cells are indeed proliferative, which could at least partially account for the mild

increase in cell number (1.6X) observed in this time window (Fig.2E).
Moreover, they say that the few proliferative cells have no specific localization within the buds, whereas it seems to me that green proliferative cells are mainly found in the middle of the mammary bud and not at the periphery (Fig.2F, also confirmed in Fig. S2B with EdU staining). I think the authors should zoom in and focus on the mammary bud, cropping out the surrounding mesenchyme. In addition, I do not see the reason for having every measurement compared to the epidermal cells; even if the epidermis cells proliferate more rapidly that the mammary cells, this does not mean that mammary growth is merely driven by cell influx, as proliferative cells are clearly present within the bud.

2- When the authors measure cell polarity by Golgi positioning, they interpret the data as proofs for the existence of cell migration within the mammary bud. I would argue that this is circumstantial evidence and that only tracing the epidermal cells that migrate into the mammary bud would conclusively prove that cell influx is a major driver of bud growth. More specific remarks:
- the nuclear sphericity (Fig. 3C) is directly correlated to the cell sphericity (Fig. 2C), so if one is lower, the other one would be lower too, but a lower sphericity in the mammary epithelial cells than in the epidermal cells does not necessarily mean that mammary "cells are deformed" (page 9).
- the 3D surface rendering is not obvious in Fig. S3A.
- I do not see what Fig. 3D conveys, I would remove it.

3- The so called "ring cells" are epidermal cells surrounding the developing mammary bud. The fact that they show strong actomyosin staining indicate that they are under tension, but this cannot be translated in the conclusion that they have a role in the invagination process, simply based on the measurement of their concentric polarity.
- at page 13, it is unclear which cells are called "mammary gland basal cells", as at this stage no specific basal marker is expressed by the cells at the periphery of the bud. Moreover, it is unclear what is defined as their apical domain in Fig. 5A.
- at the E13 stage in the neck site (Fig. 5B), the actomyosin network is localized basally and not where it would be expected if it mediated neck constriction, as suggested by the authors. I cannot really appreciate a difference in F-actin and pMLC staining at the neck region in Fig. 5B and 5C, whereas the authors claim that the staining has become uniform by E13.5: "High levels of both F-actin and pMLC persisted at same locations, as well as at the site of the prospective bulb neck at bud (E13.0) stage (Fig. 5B). At bulb (E13.5) stage, when ring cells had disappeared, the epidermal cells displayed uniform staining of both F-actin and pMLC (Fig. 5C)".
- In Fig. 5E, expression of NMIIA in the neck is not visible.
Overall, the entire Fig. 5 provides circumstantial evidence to explain a role for the actomyosin network in mammary bud invagination, which is not really demonstrated in this work; I am not sure Fig. 5 has a place as a main figure; maybe it should be moved in Supplementary Material?

4- The functional data come from the analysis of MyosinIIA cKO mice; this is an interesting experiment, but there are again several overstatements and overinterpretation of data, driving wrong conclusions: while it is true that invagination and neck formation are impaired or at least delayed in the mutant, this does not demonstrate the essential role of NMIIA in ring cells, as proposed by the authors. Indeed, as stated in point 3 above, I could find no evidence that ring cells mediate the invagination and neck formation processes.
- F-actin and pMLC expression is not reduced in the KO context at all developmental stages as stated at page 14: "Analysis of the actomyosin network showed reduced F-actin and pMLC levels at all stages analyzed (Fig. 6A-C, S5A-C)." These proteins appear less expressed only at E12.5 (Fig. 6A), whereas later on the actomyosin staining appear stronger in the KO (see phalloidin IF at E13.5 in Fig. 6A and in Fig. S5), probably due to the fact that expression is retained. By the way, Fig. 6A-C

and S5A-C represent the same time points and markers, thus they are redundant and do not provide additional information.
- at page 14 we read "conditional deletion of NMIIA leads to diminished contractile actomyosin network and causes arrest of ring cell function". Where is this shown?? Which data support this statement?
- the phenotype of the Myh9 cKO in affecting the epidermal contact area in Fig. 7C-D appears to be already present at E12.5, when we can appreciate a significantly higher contact area (Fig.7D), as well as shorter buds (Fig.7E) in the mutant. What happens before E11?
- The lack of difference in bud volume between wt and KO mice shown in Fig. 7F does not necessarily mean that there is no delay in mammary bud growth in the mutant. Indeed, the growth of all 5 buds in Myh9 KO embryos appears more synchronous in the SEM images in Fig. 7B; is this true? It would be important to measure the bud length and volume in all buds and not only in #3.
- To further evaluate the role of NMIIA in mammary morphogenesis and branching, it would also be interesting, if at all possible, to let the KO mice survive until birth and analyze the branching of the mammary primordia. An inducible Cre line to induce the KO in a timely manner may be a way to test this hypothesis. Incidentally, in the abstract, we read "the deletion of NMIIA impairs invagination resulting in abnormal mammary gland shape". The authors cannot make such a statement if they did not analyze branching at birth.

Minor remarks:

- I would remove Suppl. Fig.2A as it is the very same image as Fig.2F, with the only difference of Hoechst staining. The same is true for Suppl. Fig.2C and Fig.2A.
- At line 180, the sentence regarding the localization of the cells in S/G2/M phases refers to Fig. 2F, not 2A.
- It seems very difficult to analyze differences between E12.25 and E12.5. In Material and Methods, the authors should describe how they perform the experiment to be so precise in time.
- At E13.5, they say that ring cells had already disappeared. Therefore, in Fig.4D, they should not call the cells located in the neck region as "ring cells".
- In the legend to Fig.3 (line 856), the names of the markers are inverted. Please change to: "Cell nuclei and epithelial cells are stained with Hoechst (cyan) and EpCAM (white), respectively."

In conclusion, I find this work interesting but very speculative, as illustrated by the very long discussion (7 pages) where many exciting hypotheses are proposed, but little has been tested in the context of this work.

INSTITUTE OF BIOTECHNOLOGY
HiLIFE UNIT

March 31, 2021

JCB manuscript #202008062

Kenneth Yamada, MD, PhD
Editor
Journal of Cell Biology

Andrea L. Marat, PhD
Senior Scientific Editor
Journal of Cell Biology

Dear Drs. Marat and Yamada,

Please, find attached our revised manuscript entitled "Cell influx and contractile actomyosin force drive mammary bud growth and invagination" by Ewelina Trela *et al*. We appreciate the positive reviews and helpful suggestions provided by the reviewers. We are grateful for the editorial guidelines on the most important revision experiments, as well as the extra time allowed for revision as that allowed us to establish live imaging of developing mammary primordia.

We have addressed all the major criticism raised by the reviewers and believe that the manuscript has improved substantially. We have added key data strengthening our previous conclusions on the characteristics of the epidermal contractile ring surrounding the mammary bud and the role of NMIIA therein. Importantly, we now provide live imaging data on two morphogenetic processes: placode formation and "ring cell" activity during bud invagination. The new imaging set-up was established with support of Dr. Clémentine Villeneuve from the Wickström lab, and hence we have included one new author in the manuscript. All authors approve this change. We have also shortened the manuscript as requested. However, due to a wealth of new data (and since we were not asked to remove any of the existing data), we slightly exceed the limit of 40 000 characters (current character count is 40 655). We hope this is acceptable under these circumstances.

Below we provide a point-by-point response to each reviewer's comments. We hope that with these changes you will find our manuscript suitable for publication in *Journal of Cell Biology*. We believe that our study will be of great interest to scientists in several fields including cell and developmental biology, regenerative medicine, and breast cancer.

Thank you for considering this manuscript for publication. We look forward to hearing from you.

Yours sincerely,

Marja Mikkola

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Reviewer 1:

1. Page 7: The statement "these results indicate that during early mammogenesis the gland slowly grows and rapidly changes its shape..." do not seem consistent with the data presented in Figure 1D and E. Figure 1D shows that the volume significantly increases from E11.5 to E12.5 and from E12.5 to E13.5, and Figure 1E shows that sphericity only increases significantly from E11.5 to E12.5. Given these results, how can the growth be described as slow and the change in geometry be described as fast? Significant changes in sphericity and volume are observed over the same development time period (E11.5 to E12.5).

Response:
We fully agree with the reviewer that our previous statement was not accurate and have changed the text as follows: "Quantification showed that the volume increased by 2.5-fold between placode and bulb stages (Fig. 1 D) while its shaped changed from relatively flat into a round sphere (Fig. 1 E)."

2. Page 10: The statement "We considered that cells presumed to migrate towards the mammary gland placode would have an angle of less than 90 degrees..." is confusing. How was this criterion established? Couldn't cells with an angle of less than 90 degrees migrate away or tangential to the placode?

Response:
We apologize for the confusion. In this analysis we assessed the angle between cell vector (defined by nucleus-to-Golgi polarity) and cell nucleus-to-center of the placode. As mammary epithelial cells we considered only those cells that are located within the placode (placode defined by tissue morphology). Given that this analysis is a snapshot of the cells' polarity, we considered this as a binary analysis: either a cell is heading towards the placode center, or it is not. Indeed, our new live imaging data show that placode and non-placode cells do not differ in the straigthness of the track (although they do differ in many other cell movement parameters!), and therefore we feel that such a binary analysis (when analyzing fixed cells' polarity) remains valid. Therefore, any cell that had an angle less than 90 degrees was considered to have the polarity facing center of the placode and thus potentially migrating towards the primordium. A cell that had angle more than 90 degrees considered to have polarity facing away from the center of the placode. It should be mentioned though that in the revised manuscript, the statistics was done on the new Rose plots (see below).

3. Figure 3E and F are difficult to interpret. Firstly, the white arrows used to denote the polarity of cells are hard to read. It would be helpful if these were replaced with the vector format that is used in supplementary figure 3C. Also, the results in Figure 3F would be easier to interpret if they were plotted using the same format as the rose plot shown in Figure 4G. Lastly, the conclusion that cells are migrating into the placode would be best supported by cell trajectories obtained using live imaging, although this may not be feasible. If live-imaging is not feasible, it may be useful to reanalyze existing data to observe any migration occurring in the Z direction.

Response:
We thank the reviewer for pointing out these problems. We have changed arrows to vector format. Additionally, we have replaced Figure 3F with a rose plot.
Indeed, high-resolution live imaging of mammary placodes has turned out to be very challenging and our (numerous) previous attempts to image E11.0 - E11.5 mammary explants have been unsuccessful. Here, we decided to image whole embryos (as in Miroshnikova et al., 2018), a

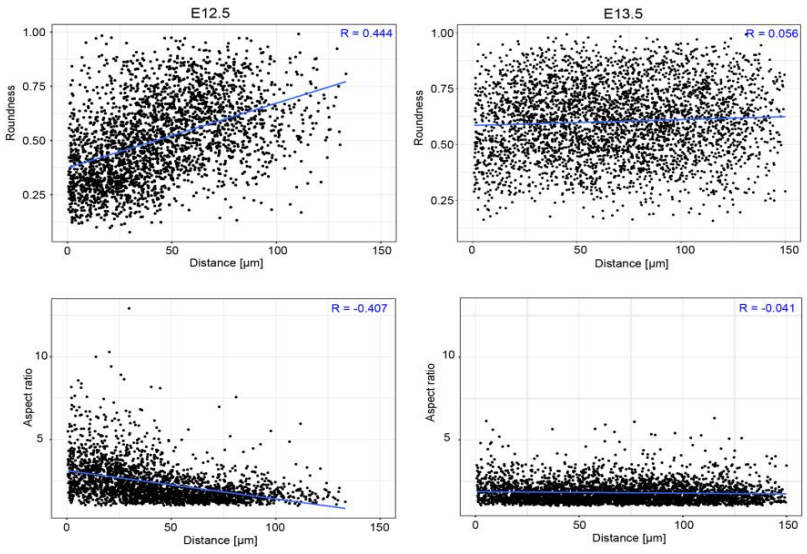
protocol that allows imaging up to 6 hours. We utilized K17-GFP; Fucci, mKO2 mouse model – red fluorescence (Fucci, mKO2) allows tracking of nuclei in K17-GFP cells which is exclusively expressed in the epithelial compartment of the skin. These new data confirm our conclusions that cell migration is the major driver of mammary placode formation. We provide new results showing cells tracjectories in Figure 3H and escape angle analysis in Figure 3I.

4. Page 10: The phrase "these cells showed a honeycomb-like alignment" is confusing. Doesn't the honeycomb pattern result from orientation of the cell borders/shape of cells? The statement would be easier to interpret if it stated that cell-cell borders formed a honeycomb pattern, which indicated that cells had a hexagonal shape.

Response:
Thank you for the suggestion. We agree that honeycomb pattern results from orientation of the cell borders/shape of cells and the phrase we used might be confusing. Therefore, we have altered text according to your suggestion; "At early placode stage (E11.25), there was no obvious difference between placodal and epidermal cells."

5. Page 11: The authors term the cells surrounding the mammary hillock "ring cells" due to "their particular appearance and arrangement." A more quantitative description of these cells here could be useful for the rest of the paper. For example, does the term ring cell refer to specific cell aspect ratio? Or a distance out from the hillock? Later, when the authors describe the cells disappearing, it would be useful to be able to refer to a specific quantitative reason why cells are no longer termed ring cells.

Response:
This is an excellent point, and we feel that the scientific community would benefit from more precise definition of ring cells. In the original manuscript, we provided 3D cell volume and sphericity data on these cells (Fig. 4D). We now complement these analyses with cell aspect ratio and cell roundness analyses, which show a sigficant difference between the ring cells and keratinocytes further away from the bud border. To obtain these data, we turned to 2D analysis of optical sections, which also allowed us to quantify cells in Myh9 cKO mutants. The limitation of this approach is that it produces inherently noisy data, because in a given optical section, cells are sectioned at random z positions (we did exclude very small "cells"). In return, this approach allows analysis of large amounts of cells. When plotted against the distance from the bud border, we observed a gradual change in both aspect ratio and roundness indicating that this approach cannot identify a definite "borderline" distance for a ring cell (see adjoining Figure, stage E12.5, provided here for reviewing purposes).



It should be also mentioned that

inspection of the 3D whole-mounts has revealed that the zone of ring cells may be more pronounced on one side of the bud than the other, so not all 'noise' is produced by the analytical approach chosen. For statistical analysis, we chose to use two cell populations that are clearly enriched either for the ring cells (0-30µm distance from bud border) and 'regular' control kerantinocytes (70-100µm distance from bud border). These new data are presented in Figure 7.

6. Figure 4: The plots in Figure 4D have a bar that represents ring cells at stage E13.5 even though the authors acknowledge that "At E13.5, when ring cells had disappeared...". Are the "ring cells" at E13.5 in these plots actually the "neck cells" that the authors refer to in the corresponding text?

Response:
The reviewer is absolutely correct. The bar marked "ring cells" in Figure 4 D does represent neck cells at stage E13.5. We apologize for our mistake and have corrected the text accordingly.

7. The authors make two claims about the Myh9 cKO condition: "Ring cells...were less pronounced than controls..." and "reduced F-actin and pMLC levels at all stages analyzed (Fig. 6A-C, S5 A-C.)". Quantification is needed to confirm these conclusions. Specifically, a metric is needed for identifying how pronounced the ring cells are. Maybe the Feret diameter or aspect ratio would be helpful for this analysis. Also, a plot comparing the intensity of F-actin and pMLC in the control and cKO condition is required.

Response:
We agree with the reviewer; these are important points to strenthen our conclusions on the Myh9 cKO phenotype. Because the ring cells are very tightly packed and thin, it is not possible to perform reliable 3D cell shape analysis on them without sparse labeling that allows rendering of individual cells (as we did for wt cells shown in Fig. 4) – an approach not possible to combine with the conditional Myh9 deletion. Therefore, to compare ring cells in Myh9 cKO condition to their wild type littermates we have utilized cell shape analaysis in 2D from optical sections of the whole-mount specimen to analyze roundness and aspect ratio (see previous response). The result showed that at E12.5 there is a modest, yet statistically significant difference between ring cells in Myh9 cKO and control littermates (but not in control keratinocytes). At E13.5, the cells surrounding the mammary bud continue to show the characteristic ring cell morphology in Myh9 cKO embryos, whereas in control embryos, they are no longer visible around the bud. Results of this analysis can be found in a new Figure 7.

Additionally, to support our conclusion of reduced F-actin and pMLC levels, we have quantified the intensities of these markers in the controls and Myh9 cKO mutants. These new results show a significant difference between Myh9 mutants and controls – data are presented in new Figure 8. Together with the cell shape analyses, these data imply that morphology, but in particular functionality of the ring cells are compromised in Myh9 cKO embryos. We have revised the text accordingly.

8. The results in Figure 7B and the statement "One day later, after invagination had occurred, the protrusion was no longer evident (Fig. 7 B)" are confusing. Adding a schematic that clearly emphasizes the tissue layers that are observed in the SEM data as compared to the immunofluorescence staining would be helpful.

Response:
We agree with the reviwer that mammary bud protrusion at E13.5 in Myh9 cKO condition in former Figure 7 B (current 9B) may not be clearly evident. In order to make this phenomenon more visible

for the reader, we provide a video (Video 4) with 3D surface rendering of epidermis and mammary bud.

9. The authors reference Figure 7G in the main text, but Figure 7G does not exist. Do they mean Figure 7F?

Response:
Thank you for spotting this mistake. We apologize for it and have corrected it.

10. Related to a previous comment, quantification is needed to support the statement "ring cells become further pronounced (Fig. 7 B)"

Response:
We appriciate the interest on ring cells and also realize that making detailed statements on ring cells based on SEM images is not valid as quantifications are not possible. Therefore, we have omitted this statement on the revised manuscript. Instead, we provide quantitative analysis of ring cells in Myh9 cKO and their littermates as detailed above.

11. In the discussion, the authors state that "Importantly, their morphology and contractility were severely impaired upon conditional deletion of NMIIA..." Quantification of the morphology of keratinocytes in both the control and the cKO condition is required to support this claim. Otherwise, the authors are overstating the findings of this study.

Response:
As detailed above, we have analyzed the shape, and intensity of phalloidin and pMLC stainings and believe that these new data better support our conclusions.

Reviewer 2

1. Some headings and statements (e.g. The first subtitle "reveals a role for cellular hypertrophy") are a bit misleading as they give the impression that hypertrophy is a major mechanism whereas the data and final conclusion of this section of the results show it is minor. The authors should present this finding more confidently. Suggest removing the term hypertrophy from the title and adjusting the subheading to be consistent with the data and conclusion.

Response:
This is a valid point. According to the reviewer's suggestion we have revised the subtitle to: "Cellular hypertrophy plays a minor role in early mammary development". This suggestion also encouraged us to change the title of the manuscript.

2. Figure 7 is the most important part of the paper but the writing in the Results section is extremely hard to follow - in its current form it is unclear where the control or the mutant is being described. Lines 303-309 definitely need rewriting.

Response:
Thank you for your suggestion. We agree that lines 303-309 were very unclear and have clarified the text as follows: "Scanning electron microscopy revealed that at E12.5, mammary primordia were elevated above surface epithelium in both control and Myh9 cKO embryos (Fig. 9 A). One day later, after invagination had occurred, the protrusion was no longer evident in controls, whereas in

Myh9 cKO mutants, mammary primordia remained elevated above the surface epithelium (Fig. 9 B). 3D surface rendering of EpCAM stained specimen further confirmed this conclusion (Fig. 9 C, Video 4). Next, we used these 3D renderings to quantify the epidermal contact area and invagination of the mammary rudiment from placode (E11.5) to bulb (E13.5) stage. At placode stage, no significant difference was observed between the controls and mutants (Fig. 9 D-E). In control embryos, the epidermal contact area steadily decreased in controls during the invagination process, whereas in Myh9 cKO embryos, this was significantly less pronounced (Fig. 9 D).The invagination, measured as the depth of the mammary rudiment, was also substantially impaired in Myh9 cKO at hillock stage (E12.5-E13.0), but not anymore at bulb (E13.5) stage (Fig. 9 E)."

3. The figure legends are very detailed but give little guidance as to the main points the reader is supposed to look at in the images - in several arrows are in the figures but there is no explanation in the legend as to what they are indicating.

Response:
This is a very valid point and we apologize for omitting these important details in the figure legends. We have revised all figure legends and hope that they now better guide the reader to the main points.

4. The discussion would benefit from being shortened a little. For example, suggest cutting out lines 344-351

Reponse:
As suggested by reviewers 1 and 3, and also due to the JCB length limitation, we have substantially shortened the discussion from 7 pages to 4.5 pages.

5. The term ring cells doesn't convey the supracellular nature of this structure or its proposed mechanism- maybe something like - epidermal contractile ring?? (Just a suggestion).

Response:
Thank you for your suggestion. When discussing the ring cells as a collective, the term proposed seems approriate, yet there are occasions, in particular when referring to individual cells, where we find it more practical to call them ring cells.

6. Minor points:

a. Line 88 "The First...." Make word "first" lowercase

Reponse:
Thank you for finding this out. We have corrected the text accordingly.

b. Line 132 "enlarged" - this term is too vague and does not apply to the first part of figure which shows reduced surface area - suggest use "enlarged volume" and refer specifically to the reconstruction part of the figure

Response:
Thank you for your suggestion. We have modified the text as follows: "At the hillock stage, the mammary primordium had already enlarged in volume, became rounded and deepened more prominently into the underlying mesenchyme (Fig. 1 B-C)."

c. Line 400 "describe and undescribed" needs rephrasing

Response:
We have rephrased this sentence to: "Here, we delineate an undiscribed mechanism of organ invagination driven by a rim of contractile cells around the invaginating multilayered tissue."

d. Line 855 (Fig 3 legend) - need to reverse the order of Epcam and Hoechst in sentence: of "Cell nuclei and epithelial cells are stained with EpCAM (white) and Hoechst (cyan), respectively.

Response:
Thank you for pointing this out. We have revised all figure legends and have hopefully managed to correct all mistakes.

e. Fig 5C and throughout the text don't use "expression" - it's just levels by immunofluorescence- perhaps "intensity" is safer as relocalization rather than upregulated gene expression seems more likely to account for it.

Response:
Thank you for this suggestion. In the revised manuscript, we have used intensity as reference to levels of immunofluorescence.

f. Throughout text check if use of mammary gland is appropriate - in some cases just "mammary" is sufficient e.g. mammary fate and in others perhaps "primordium" or "rudiment" might be better than "gland" as its glandular nature is not yet acquired at E12-15.

Response:
We have refined the text according to the reviewer's suggestion and have replaced the word gland by rudiment or primordium whenever appropriate.

Reviewer 3

1- The authors study the dynamics of volume gain during mammary growth from placode to bulb. Based on the data presented in Fig. 2, they claim having "compelling evidence" for a "proliferation-independent" growth mechanism. I disagree with this conclusion as in Fig. 2G we can appreciate that 6-9% of the cells are indeed proliferative, which could at least partially account for the mild increase in cell number (1.6X) observed in this time window (Fig.2E).

Response:
Thank you for raising this issue for further discussion. We agree with the reviewer that the proliferating cells may have a small contribution to the growth of the mammary rudiment and have revised the text accordingly. It is, however, difficult to estimate exactly how much the dividing cells might contribute, because the length of the cell cycle of surface epithelial cells is unknown at these developmental stages. The recent paper from Blanpain group has estimated that cell cycle length at P0 is approximately 1.2 days {Dekoninck, 2020 #107}. If the same applies to earlier stages, we would expect to observe increase in the percentage of proliferating cells from placode to hillock, and further more from hillock to bulb stages (provided that proliferation would be the only means how the cell number increases) between those stages. Yet, our data indicate the decrease in percentage of S/G2/M cells from 9% to 6% suggesting that suppresion of cell proliferation is key characteristic of early mammary morphogenesis.

Moreover, they say that the few proliferative cells have no specific localization within the buds, whereas it seems to me that green proliferative cells are mainly found in the middle of the mammary bud and not at the periphery (Fig.2F, also confirmed in Fig. S2B with EdU staining). I think the authors should zoom in and focus on the mammary bud, cropping out the surrounding mesenchyme. In addition, I do not see the reason for having every measurement compared to the epidermal cells; even if the epidermis cells proliferate more rapidly that the mammary cells, this does not mean that mammary growth is merely driven by cell influx, as proliferative cells are clearly present within the bud.

Response:
Thank you for this comment. Figures 2 F and S2B (current S2C) are derived from confocal images and represent only one optical section omitting the rest of the image, therefore, might be misleading and we apologize for the confusion. We provide 3D videos of mammary primordium from Fucci transgenic cell cycle reporter mouse and mammary primodrium stained with EdU in which surrounding mesenchyme has been cropped out for better visualization for reviewing purposes. These videos show that cells in G2/S/M phases have no specific localization within the bud. However, since we have not quantified these cells with respect to their location, we prefer to omit the sentence stating that they are randomly distributed.

The reviewer was also wondering why we compare mammary cell characteristics to epidermal cells. The reason is that surface epithelial (epidermal) cells are the progenitors of mammary epithelial cells, therefore, we feel that they are the most appropriate control group. In many figures, we also provide comparisons of mammary cells accross different developmental stages.

2- When the authors measure cell polarity by Golgi positioning, they interpret the data as proofs for the existence of cell migration within the mammary bud. I would argue that this is circumstantial evidence and that only tracing the epidermal cells that migrate into the mammary bud would conclusively prove that cell influx is a major driver of bud growth.

Response:
The reviewer is absolutely correct in that analysis of cell polarity is more of circumstantial evidence than direct proof and we agree that live imaging would provide more conclusive evidence on the role of cell migration. Over the past couple of years, we have learnt that live imaging of early embryonic explants at cellular resolution is very challenging. Therefore, we are extremely delighted to provide novel confocal time-lapse imaging data on nascent mammary placodes. The analysis of the cell behaviors confirmed that mammary cells migrate toward the placode providing strong evidence that cell influx is the main driver of mammary placode formation. The new results are presented in Figure 3 G-I.

- the nuclear sphericity (Fig. 3C) is directly correlated to the cell sphericity (Fig. 2C), so if one is lower, the other one would be lower too, but a lower sphericity in the mammary epithelial cells than in the epidermal cells does not necessarily mean that mammary "cells are deformed" (page 9).

Response:
Thank you for poitining this out. We agree that the term “deformed” might not accurately describe change is mammary epithelial cells shape observed as change in nuclear shape, hence, we have revised the text and instead use the term strain. Yet, our data clearly show that mammary placode cells differ from epidermal cells.

- the 3D surface rendering is not obvious in Fig. S3A.

Response:
We apologize for not making this point obvious. We now show the rendered cells also without EpCAM staining to better visualize them.

- I do not see what Fig. 3D conveys, I would remove it.

Response:
With this figure, we aimed to show an overview of the tissues with the indicated stainings. We have now placed the figure in the supplements for the interested readers.

3- The so called "ring cells" are epidermal cells surrounding the developing mammary bud. The fact that they show strong actomyosin staining indicate that they are under tension, but this cannot be translated in the conclusion that they have a role in the invagination process, simply based on the measurement of their concentric polarity.

Response:
Indeed the reviewer is correct in that stronger evidence on the behavior of the ring cells would strenghten our conclusions. To support the conclusion that ring cells have role in the invagination process we present novel live imaging data of mammary primordium (Figure 5). First, we show that epidermal contact area of mammary bud decreases during the imaging session indicating an ongoing invagination process during ex vivo culture. Importantly, vector analysis showed that ring cells have opposing directions and clearly behave differently from the epidermal cells that are further away from the bud. This analysis showed that majority of ring cells vectors are at 45-90 degrees with respective to the bud, indicating that they move in a circular fasion during the invagination process.

- at page 13, it is unclear which cells are called "mammary gland basal cells", as at this stage no specific basal marker is expressed by the cells at the periphery of the bud. Moreover, it is unclear what is defined as their apical domain in Fig. 5A.

Response:
Thank you for pointing this out. With basal cells we refer to cells whose basal domains are located at the epithelial-mesenchymal border (i.e. basement membrane) which we evaluate based on the EpCAM staining that is used to mask the mammary bud. Hence, in this context, the definition of 'basal cell' is morphological and independent of any cell lineage markers. To clarify this, the sentence now reads: "In addition, high F-actin intensity marked basal (proximal to basement membrane) and apical (distal to basement membrane) domains of the basally-located mammary cells."

- at the E13 stage in the neck site (Fig. 5B), the actomyosin network is localized basally and not where it would be expected if it mediated neck constriction, as suggested by the authors. I cannot really appreciate a difference in F-actin and pMLC staining at the neck region in Fig. 5B and 5C, whereas the authors claim that the staining has become uniform by E13.5: "High levels of both F-actin and pMLC persisted at same locations, as well as at the site of the prospective bulb neck at bud (E13.0) stage (Fig. 5B). At bulb (E13.5) stage, when ring cells had disappeared, the epidermal cells displayed uniform staining of both F-actin and pMLC (Fig. 5C)".
- In Fig. 5E, expression of NMIIA in the neck is not visible.

Response:

We have not analyzed the behavior of the neck cells after invagination, and although interesting, we feel that their role is likely to be more relevant for the maintenance of the invaginated bud rather than the invagination process per se. We have omitted all quantitative statements on cells in the neck region unless supported by data that has been quantified.
We agree that expression of NMIIA in the neck was not clearly visible in Figure 5E and apologize for that. We have placed these low magnification figures in the supplement and instead, provide higher resolution images of NMIIA expression at E13.5 in the main figure (Fig. 6D). More importantly, we have quantified the intensity of NMIIA (Fig. 6E). These new data show that NMIIA intensity in the neck is lower than in the epidermis, yet substantially higher than the bud proper.

Overall, the entire Fig. 5 provides circumstantial evidence to explain a role for the actomyosin network in mammary bud invagination, which is not really demonstrated in this work; I am not sure Fig. 5 has a place as a main figure; maybe it should be moved in Supplementary Material?

Response:
We appreciate the reviewer's concern on the role of the actomyosin network in mammary bud invagination. The point of Figure 5 was to show phalloidin and pMLC staining in wild-type embryos which motivated us to functionally assess the role of NMIIA in bud invagination. We agree that it was partially overlapping with Figure 6 (current Figure 8) where we showed the same stainings in Myh9 cKO and their control littermates. To avoid unnecessary overlaps, the revised Figure 8 (former Fig. 6) only includes the most relevant figures on Myh9 cKO and their control littermates, and the rest has been moved to supplements. Importantly, we have quantified the intensity levels of F-actin and pMLC (Figure 8). These new data show that deletion of Myh9 leads to reduced activity of actomyosin network. Additionally, we have analazyed the shape of ring cells (roundness and aspect ratio). This quantification showed that ring cells in Myh9 cKO are not only impaired at E12.5, but that they persist at E13.5 whereas in control littermates ring cells are no longer discernible at E13.5 (Figure 7).

4- The functional data come from the analysis of MyosinIIA cKO mice; this is an interesting experiment, but there are again several overstatements and overinterpretation of data, driving wrong conclusions: while it is true that invagination and neck formation are impaired or at least delayed in the mutant, this does not demonstrate the essential role of NMIIA in ring cells, as proposed by the authors. Indeed, as stated in point 3 above, I could find no evidence that ring cells mediate the invagination and neck formation processes.
- F-actin and pMLC expression is not reduced in the KO context at all developmental stages as stated at page 14: "Analysis of the actomyosin network showed reduced F-actin and pMLC levels at all stages analyzed (Fig. 6A-C, S5A-C)." These proteins appear less expressed only at E12.5 (Fig. 6A), whereas later on the actomyosin staining appear stronger in the KO (see phalloidin IF at E13.5 in Fig. 6A and in Fig. S5), probably due to the fact that expression is retained. By the way, Fig. 6A-C and S5A-C represent the same time points and markers, thus they are redundant and do not provide additional information.
- at page 14 we read "conditional deletion of NMIIA leads to diminished contractile actomyosin network and causes arrest of ring cell function". Where is this shown?? Which data support this statement?

Response:
In order to address these issues, we have analyzed the intensity levels of F-actin and pMLC in Myh9 cKO mutants and control littermates. This quantification strengthness our previous finding as

significant differences in intensity levels were found in F-actin and pMLC at both stages analyzed. These data are shown in Figure 8.
Overall, to provide further evidence for the role of NMIIA in ring cells and invagination, we present several new piece of data, as detailed above and summarized here. We 1) show live imaging of ring cells revealing that they move in a circular fashion ("around the bud") during the invagiantion process; 2) have quantified the shape of ring cells and show that Myh9 deficiency affects their shape at E12.5 and unlike in controls, ring cells are maintained in Myh9 cKO at E13.5; and c) have quantified F-actin and pMLC intensities in Myh9 cKO and control embryos showing the reduced levels in Myh9 cKO mutants. We believe that collectively, these data provide robust evidence for the role of ring cells and NMIIA therein in the invagination process.
We have added new Figures to report these new data. In its current manuscript, the main figures contain much less overlap and only show the most relevant stainings/magnifications to accompany the quantifications.

- the phenotype of the Myh9 cKO in affecting the epidermal contact area in Fig. 7C-D appears to be already present at E12.5, when we can appreciate a significantly higher contact area (Fig.7D), as well as shorter buds (Fig.7E) in the mutant. What happens before E11?

Response:
Thank you for your suggestion. To answer your question we have collected Myh9 cKO and control samples at E11.5 and have quantified the epidermal contact area, bud depth and bud volume. We found no difference between Myh9 cKO and control samples in any of the three parameters indicating that Myh9 cKO phenotype appears between E11.5 and E12.5. The results are included in Figure 8 C-F.

- The lack of difference in bud volume between wt and KO mice shown in Fig. 7F does not necessarily mean that there is no delay in mammary bud growth in the mutant. Indeed, the growth of all 5 buds in Myh9 KO embryos appears more synchronous in the SEM images in Fig. 7B; is this true? It would be important to measure the bud length and volume in all buds and not only in #3.

Response:
It is well-established that mammary primordia develop asynchronously and sequencially as follows: 3 first, followed 4, then 1 and 5, and number 2 as last (Veltmaat et al., Dev Dyn 2004). Given this asynchrony, we considered it essential to always analyze the same mammary bud. We chose to focus on mammary bud 3 due to its central position and accessibility, which turned out to be a very good decision as the live imaging set-up that turned out to be successful was particularly well suited for mammary rudiment 3. Unfortunately, we did not have the resources to perform a detailed analysis of all five Myh9 cKO mammary rudiments due to a major collapse in our Myh9 floxed colony. Yet, we see no reason why our findings on mammary bud 3 would not apply also to others.

- To further evaluate the role of NMIIA in mammary morphogenesis and branching, it would also be interesting, if at all possible, to let the KO mice survive until birth and analyze the branching of the mammary primordia. An inducible Cre line to induce the KO in a timely manner may be a way to test this hypothesis. Incidentally, in the abstract, we read "the deletion of NMIIA impairs invagination resulting in abnormal mammary gland shape". The authors cannot make such a statement if they did not analyze branching at birth.

Response:

This is an excellent suggestion and we agree with the reviewer that dissecting the role of NMIIA in mammary branching would be highly interesting. However, to appropriately evaluate the role of NMIIA in branching morphogenesis would require a completely different set of experiments and necessitate the analysis of multiple stages after the bulb stage. As discussed with the Editors, this would constitute a study of its own that is beyond the scope of this manuscript. We hope to address this question in the future.
As suggested, we have revised the abstract and it now reads: "Furthermore, we show that conditional deletion of non-muscle myosin IIA (NMIIA) impairs invagination resulting in abnormal mammary bud shape."

Minor remarks:
- I would remove Suppl. Fig.2A as it is the very same image as Fig.2F, with the only difference of Hoechst staining. The same is true for Suppl. Fig.2C and Fig.2A.

Response:
We respectfully disagree with the reviewer. We believe that separate images with Hoechst staining better reveal the amount of cells that are present in both epithelial and mesenchymal tissues and showing cell density might be of interest for the reader.

- At line 180, the sentence regarding the localization of the cells in S/G2/M phases refers to Fig. 2F, not 2A.

Response:
We thank the reviewer for spotting this mistake. It has now been corrected.

- It seems very difficult to analyze differences between E12.25 and E12.5. In Material and Methods, the authors should describe how they perform the experiment to be so precise in time.

Response:
Our lab has a long track-record on working with early embryonic stages (e.g. Närhi et al., Dev Biol 2008; Voutilainen et al., PNAS 2012 and PloS Genet 2015; Ahtiainen et al., Dev Cell 2014 and J Cell Biol 2016). We have amended Material and Methods to indicate that the main morphological criteria used in the study were limbs and other external criteria. In a regular light-dark cycle, mice tend to mate around midnight (time of mating is considered E0.0), and we take this as the starting point to decide the time of the day when to sacrifice the pregnant dams. It should be emphasized, however, that each embryo in every litter is staged individually according to the morphological criteria. In the current study, all embryos were stages by one researcher (Ewelina Trela) to ensure consistency in embryo staging.

- At E13.5, they say that ring cells had already disappeared. Therefore, in Fig.4D, they should not call the cells located in the neck region as "ring cells".
- In the legend to Fig.3 (line 856), the names of the markers are inverted. Please change to: "Cell nuclei and epithelial cells are stained with Hoechst (cyan) and EpCAM (white), respectively."

Response:
We apologize for these mistakes. We have modified Figure 4 D and labeled them as neck cells at E13.5. We have revised all Figure legends and hopefully have avoided mistakes.

May 5, 2021

RE: JCB Manuscript #202008062R

Dr. Marja L Mikkola
University of Helsinki
P.O.Box 56
University of Helsinki 00014
Finland

Dear Dr. Mikkola:

Thank you for submitting your revised manuscript entitled "Cell influx and contractile actomyosin force drive mammary bud growth and invagination". It was re-assessed by two of the three original expert reviewers, whose reports are appended below. As you can see, one reviewer was satisfied with the revisions while the other reviewer had some specific concerns that could be resolved by text revisions. Please consider these points carefully and return a final revised version of your manuscript that meets our formatting guidelines (see details below) so that we can evaluate it at the senior Editor level and, we hope, proceed to final acceptance for publication.

We look forward to receiving final revisions to the text of this interesting study.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

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

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the

test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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

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

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

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

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

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

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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

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

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

B. FINAL FILES:

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

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

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

Sincerely,

Kenneth Yamada, MD, PhD
Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors have addressed all of the previous concerns, greatly improving the clarity and impact of their manuscript. Their findings reveal a novel mechanism for formation of the mammary anlage and illustrate a role for keratinocytes in mammary gland development.

Reviewer #3 (Comments to the Authors (Required)):

In the revised manuscript the authors have partly addressed some of my previous concerns by incorporating new data (time-lapse imaging) and better analyzing previous datasets. However, I found it pretty difficult to follow the changes compared to the former version, since they were not specified in the Rebuttal letter. We read for example: "We have now placed the figure in the supplements for the interested readers." Which supplements are they referring to? Or "We have added new Figures to report these new data". It would have been appreciated if the authors were more specific in indicating their corrections and if the new data were highlighted in the text (i.e. with a different colour).

Notwithstanding the authors' efforts, some overstatements and overinterpretations of data have remained in the revised version, leading in some instances to strong conclusions not always supported by experimental evidence.
Here below are the points that were not thoroughly addressed in the revision:

1. Regarding the point on the contribution of proliferation to bud growth and invagination, at page 8 we read: "We observed similar absence of replicating cells by analyzing proliferation with 5-ethynyl-2'-deoxyuridine (EdU) incorporation validating our conclusions from the Fucci model (Fig. S2 C)."

Both Fig. S2C and the new video 5 show several EdU+ cells at E12.5 and even more dividing cells at E13.5, so the cited sentence does not reflect the data presented. I suggest to change the sentence to: "we observed low numbers of replicating cells..."

2. In response to my point: the 3D surface rendering is not obvious in Fig. S3A, the authors replied: "We apologize for not making this point obvious. We now show the rendered cells also without EpCAM staining to better visualize them."

I could not find that: in Fig. S3, they simply swapped panels A and B, but the panels are identical to the previous figure! In addition, they kept the identical panel presenting 3D surface rendering also in Fig. 1B. I also could not find the figure without EpCAM anywhere. Please, correct.

3. The so called "ring cells" are epidermal cells surrounding the developing mammary bud. The fact that they show strong actomyosin staining indicate that they are under tension, but this cannot be translated in the conclusion that they have a role in the invagination process, simply based on the measurement of their concentric polarity.

The interpretation from time-lapse microscopy that ring cells "move in a circular fashion during the invagination process" is not evidence for a functional role of these cells in invagination. Only mutants or cell ablation experiments can properly address the function of ring cells. I understand

that such experiments would represent a whole new study, but at least, the conclusions must be tuned down throughout the article, starting with the Abstract: "ring cells - that form a contractile rim around the mammary bud and exert force via the actomyosin network (there is not real force assessment but rather a prediction).
To answer my concern about previous Fig. 5 (now Fig. 6), the authors cite results obtained in the Myh9 cKO mutant, which is not presented in the Figure...? Incidentally, in the final PDF, the figures' number is not indicated on the figure panels, making the reading pretty difficult.

4. while it is true that invagination and neck formation are delayed in the mutant, this does not demonstrate the essential role of NMIIA in ring cells, as proposed by the authors (this is more a prediction/assumption). Indeed, as stated in point 3 above, I could find no evidence that ring cells mediate the invagination and neck formation processes. Once more, I suggest that the authors tune down the conclusions of this part of the study.

5. at page 14 we read "conditional deletion of NMIIA leads to diminished contractile actomyosin network and causes arrest of ring cell function". Where is this shown? Which data support this statement?

In response to my two concerns above, the authors answered:
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In Fig. 8, Myh9 cKO ring cells show reduced F-actin at E12.5 but stronger phalloidin expression at E13.5; this confirms my original remark and does not reflect "reduced levels in Myh9 cKO mutants at all stages", as stated in the manuscript.

Finally, a minor point about precisely staging the embryos: the authors added this sentence in Materials and Methods: "the main morphological criteria used in the study were limbs and other external criteria".

I do not think that this sentence specifies how the embryos were staged. "Other external criteria" is a very vague statement, that defeats the purpose of the Methods sections, that should allow other labs to faithfully reproduce reported experiments.

In conclusion, while I find the study interesting and I think it would be important to publish it, I really wish the authors could downplay their conclusions for the points I mention and avoid overstatements.

INSTITUTE OF BIOTECHNOLOGY
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May 7, 2021

JCB manuscript #202008062 R2

Kenneth Yamada, MD, PhD
Editor
Journal of Cell Biology

Andrea L. Marat, PhD
Senior Scientific Editor
Journal of Cell Biology

Dear Drs. Marat and Yamada,

Please, find attached the second revision of the manuscript entitled "Cell influx and contractile actomyosin force drive mammary bud growth and invagination" by Ewelina Trela *et al*. We appreciate the positive comments and that all reviewers now find that the manuscript merits publication.

We have addressed the remaining criticism raised by reviewer 3. All points relevant to his/her comments are highlighted in red in the revised manuscript; the point-by-point response is presented below. We have inspected the manuscript to ensure that it adheres to all formatting guidelines. Our current character count is < 40 000.

We hope that with these changes you will find our manuscript suitable for publication in *Journal of Cell Biology*. We believe that our study will be of great interest to scientists in several fields including cell and developmental biology, regenerative medicine, and breast cancer.

Thank you for considering this manuscript for publication.
We look forward to hearing from you.

Yours sincerely,

Marja Mikkola

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Reviewer 3:

1. Regarding the point on the contribution of proliferation to bud growth and invagination, at page 8 we read: "We observed similar absence of replicating cells by analyzing proliferation with 5-ethynyl-2'-deoxyuridine (EdU) incorporation validating our conclusions from the Fucci model (Fig. S2 C)."

Both Fig. S2C and the new video 5 show several EdU+ cells at E12.5 and even more dividing cells at E13.5, so the cited sentence does not reflect the data presented. I suggest to change the sentence to: "we observed low numbers of replicating cells..."

Response:
Taking into account the suggestion of the reviewer, we have modified the text accordingly. Starting from the line 162 on page 8 of the manuscript, the corrected text reads as follows: "Similarly, we observed low numbers of replicating cells by analyzing proliferation with 5-ethynyl-2'-deoxyuridine (EdU) incorporation validating our conclusions from the Fucci model (Fig. S2 C)."

2. In response to my point: the 3D surface rendering is not obvious in Fig. S3A, the authors replied: "We apologize for not making this point obvious. We now show the rendered cells also without EpCAM staining to better visualize them."

I could not find that: in Fig. S3, they simply swapped panels A and B, but the panels are identical to the previous figure! In addition, they kept the identical panel presenting 3D surface rendering also in Fig. 1B. I also could not find the figure without EpCAM anywhere. Please, correct.

Response:
We apologize for not making our correction clear enough. In current Fig. S3A we have included three still images: first containing EpCAM staining together with placode surface rendering, and nuclear reporters, second containing EpCAM staining together with placode surface rendering and nuclear surface renderings, third containing only placode surface rendering without EpCAM staining and nuclear surface renderings. These images were done for both MECs and epidermal cells. We have now included a close-up of nuclei in the third picture in this series – we hope the reviewer finds this helpful. We have also added a sentence to Supplemental Figure 3 legend stating: Grey area in the upper picture delineates the placode. Insets are close-ups of the indicated areas. Current Fig. S3B was moved from old Fig. 3D according to the suggestion of the reviewer during first revision.

We do not understand the comment on Figure 1B. The previous review did not criticize the rendering shown in Fig. 1B in any way.

3. The so called "ring cells" are epidermal cells surrounding the developing mammary bud. The fact that they show strong actomyosin staining indicate that they are under tension, but this cannot be translated in the conclusion that they have a role in the invagination process, simply based on the measurement of their concentric polarity.

The interpretation from time-lapse microscopy that ring cells "move in a circular fashion during the invagination process" is not evidence for a functional role of these cells in invagination. Only mutants or cell ablation experiments can properly address the function of ring cells. I understand that such experiments would represent a whole new study, but at least, the conclusions must be tuned down throughout the article, starting with the Abstract: "ring cells - that form a contractile rim around the mammary bud and exert force via the actomyosin network (there is not real force assessment but rather a prediction).

Response:
As requested, we have changed the phrasing of the test in several places (new text indicated in red).

Abstract:
We delineate a hitherto undescribed invagination mechanism driven by thin, elongated keratinocytes – ring cells – that form a contractile rim around the mammary bud and likely exert force via the actomyosin network.
Summary statement:
In this study, Trela et al. delineate the cellular mechanisms governing early mammary gland development and find that its initial growth is primarily accomplished by cell migration. Moreover, ~~they show~~ they suggest that mammary bud invagination is driven by contractile cells encircling the bud – the ring cells.
Introduction:
The last two sentences were deleted in their entirety: ~~We show that growth of the mammary rudiment is mainly achieved by cell influx and to smaller extent by cellular hypertrophy and proliferation. Moreover, we report an undescribed mechanism of tissue invagination, where epithelial cells (keratinocytes) surrounding the mammary bud propagate contractile force generated by the actomyosin network to induce cell contraction and bud invagination. Conditional deletion of non-muscle myosin IIA (NMIIA) compromises invagination resulting in abnormal mammary bud shape.~~
Results:
Lines 314-316 (last sentence of Results): Taken together, our results indicate that loss of epithelial NMIIA diminishes actomyosin contractility and ~~arrests~~ suggest arrested ring cell function as the likely cause of the ~~leading to~~ impaired mammary bud invagination and neck formation.
Discussion:
Lines 405-407: In conclusion, this work provides new insights into early mammary morphogenesis by showing that mammary placodes coalesce by cell migration and ~~uncovering~~ proposes a previously undescribed invagination mechanism through a rim of contractile epithelial cells.

To answer my concern about previous Fig. 5 (now Fig. 6), the authors cite results obtained in the Myh9 cKO mutant, which is not presented in the Figure...? Incidentally, in the final PDF, the figures' number is not indicated on the figure panels, making the reading pretty difficult.

We failed to understand what the reviewer means with this comment. We have once more checked the main text (lines 252-267 on page 12) describing results presented in Figure 6 and find it accurate and correctly referring to images shown in Figure panel 6.

4. while it is true that invagination and neck formation are delayed in the mutant, this does not demonstrate the essential role of NMIIA in ring cells, as proposed by the authors (this is more a prediction/assumption). Indeed, as stated in point 3 above, I could find no evidence that ring cells mediate the invagination and neck formation processes. Once more, I suggest that the authors tune down the conclusions of this part of the study.

Response:

As suggested by the reviewer, we have toned down our conclusions in several occasions; see our response to point 3.

5. at page 14 we read "conditional deletion of NMIIA leads to diminished contractile actomyosin network and causes arrest of ring cell function". Where is this shown? Which data support this statement?

In response to my two concerns above, the authors answered:
"We have quantified F-actin and pMLC intensities in Myh9 cKO and control embryos showing the reduced levels in Myh9 cKO mutants. We believe that collectively, these data provide robust evidence for the role of ring cells and NMIIA therein in the invagination process."

In Fig. 8, Myh9 cKO ring cells show reduced F-actin at E12.5 but stronger phalloidin expression at E13.5; this confirms my original remark and does not reflect "reduced levels in Myh9 cKO mutants at all stages", as stated in the manuscript.

Response:
We had considered concerns raised by the reviewer in the first revision and ***did not state*** *that we observed "reduced levels in Myh9 cKO mutants at all stages". In the manuscript, on page 13, lines 293-297, we write: "Myh9 cKO embryos displayed a significant decrease in in F-actin intensities at E12.5 and pMLC intensities at both stages compared to control embryos indicating diminished contractile actomyosin network (Fig. 8 C-D). At E13.5, F-actin remained slightly elevated in the bud-proximal cells in Myh9 cKO embryos compared to controls, likely because of the persisting ring cells."*

Finally, a minor point about precisely staging the embryos: the authors added this sentence in Materials and Methods: "the main morphological criteria used in the study were limbs and other external criteria".

I do not think that this sentence specifies how the embryos were staged. "Other external criteria" is a very vague statement, that defeats the purpose of the Methods sections, that should allow other labs to faithfully reproduce reported experiments.

Response:
We apologize for too vaguely stating the criteria that were used for age of the embryos and that we had not reported that in all embryos younger than E12.0, somites were counted. Corrected text states as follows (pages 19-20 lines 434-437): "Embryonic ages were always assessed by the same person (E.T.) and defined based on the date of the vaginal plug, limb and craniofacial morphology, and other external criteria (Martin, 1990). For embryos younger than E12.0, the number of somites was used to stage the embryos (Theiler, 1989)."