Peer Review File

Manuscript Title: Skin cells undergo asynthetic fission to expand body surfaces in zebrafish

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Chan and coworkers presents a live imaging study of the superficial epidermal layer of the developing zebrafish. The authors show that the cells, previously considered postmitotic, undergo fission to generate cellular material for the expanding tissue. They further present data and modeling to suggest that the divisions occurs in the absence of DNA synthesis.
This is an intriguing manuscript that presents an interesting process of cell fission that could provide a rapid and effective means of tissue expansion. The imaging data is beautiful, of high quality, and convincing. The main shortcoming of the manuscript is that it describes the phenomenon of cell fission superficially and many of the quantitative data and statistical analyses do not appear robust. In particular the role of DNA synthesis is studied very superficially. Finally, while it is understandable that a full mechanism might not be fleshed out in a single study, the absence of any mechanistic analyses weakens the impact of the study.

Specific points:

1. The mean increase in SEC number in 10 dpf animals is in fact driven by 3 outlier animals, where the rest are in the range of the 8 dpf, which also likely explains why this result is not statistically significant and questions the robustness of this observation.
2. Given the data showing that unlabeled cells rapidly take over the tissue, as expected, hue balance should be quantified also at the 10 hpf time point and the data presented separately for each animal to exclude that clones arise from rare, leaky subrabasal or basal cells.
3. The data for decreased genome size is not robust as it could also be attributed to the shedding that occurs with a very high rate. The authors need to distinguish the subG1 cells that have divided versus the ones that are dying during shedding.
4. Describing the effect of HU on SEC clone size as “moderate” seems like a strong understatement. The data suggest that both the clone size distribution and the divided pool are restored to near 9hpf levels. It is also not clear why a student´s t-test is used for statistical testing of Ext. Data Fig. 4k (divided cell pool) as the authors are comparing 3 groups, so a t-test is inappropriate and a Anova-type test should be used. Also, a post hoc test to separately compare the difference between untreated and treated at 10hpf is required, as this most likely would show a significant, rather than a moderate effect of the treatment.
5. The authors need to scrutinize statistical testing throughout the manuscript and apply appropriate anova-type tests instead of t-tests when 3 or more groups are being compared.

6. Although in the abstract the authors state that the process occurs “presumably through tension-mediated activation of stretch-activated ion channels”, no mechanistic analyses are provided and no data for ion channel involvement is presented
7. The last sentence of the abstract: “This frugal yet flexible mode of cell proliferation may also be relevant to non-skin cells and disease conditions” is very speculative at this point and would be better removed.

Minor
1. It is not clear how the SEC Coverage ratio (Fig 2g) is defined and computed

Referee #2 (Remarks to the Author):

This paper by Chen and co-authors reports a fascinating new discovery. Using clonal labelling methods to mark individual outer skin cells of the zebrafish larvae they report rounds of cell division that occur in the absence of DNA replication. During a specific phase in the animals life (prior to day 21), this leads to the formation of small clones of cells in the skin with reduced DNA content. By imaging these divisions live they show that cell division is accompanied by DNA segregation, making it appear as if this is a case of cells entering mitosis without undergoing DNA replication (as occurs in fission yeast cut mutants).

Since these division events are more prevalent in larger animals, which have more surface to cover, and correlate with local differences in surface growth, they suggest that these likely contribute to the ability of the skin to cover the growing embryo. Finally, they show that these are dependent on Piezo channels using gain and loss of function drug perturbations.

The paper is well written, includes a large number of relevant experiments that are well-controlled and quantified appropriately and lots of stunning images. It promises to open up a whole new area of biology, will interest a wide audience, and would therefore be appropriate for a journal like Nature.

Before being published however there are a number of issues that should be addressed. Most could be addressed by alterations to the text, additional analyses, and a small number of experiments.

Key concerns:
1. Interestingly, previous work in Drosophila larvae (following wounding) and C. elegans (development) suggests that skin cells fuse to ensure good coverage of the animal. Therefore more needs to be done to make it clear how this type of division aids coverage of the animal with limited material as the authors suggest. In fact, by undergoing division cells need more plasma membrane not less – something might raise tension.

2. The data on Gd+++ and Yoda are exciting and should be in the main text. They imply that division is dependent on Piezo. Although not essential, it would be good to show this with a genetic

experiment. In addition, it would be good to determine the consequences of these treatments for the ability of the animal to recover from wounding.
In doing so the authors should test:
i) The apical area and height of cells in Gd+++, control and Yoda treated animals.
ii) The orientation of the long cell axis in all conditions.
iii) DNA content in all conditions. Are cells diploid in Gd+++ treated cells?
iv) How do these treatments alter the loss of these SEC cells as animals mature?
v) Why do there appear to be lots of dark clones with large number of small cells in Gd images (S6)? Is this taken into account when assessing Trunk surface area?

3. The authors should do more to test the extent to which the cells undergo a conventional mitosis before they divide. Thus, far their paper only includes a few low resolution images which suggest that nuclear division precedes cell division.

i) To do so, they should image spindles (can be done in fixed tissues), DNA (with DAPI/Hoechst or equivalent) to measure DNA content using microscopy, and Lamin or equivalent to see if the nuclear compartment boundary is lost at division. It would be great (but not essential) to know if CDK inhibitors arrest the process as would be expected.
ii) If cells assemble a spindle prior to division this could guide the orientation of the division axis.
iii) It is not clear how mitosis can proceed normally if chromosomes are not able to form bipolar attachments. Is mitosis as a result slower in these divisions than it is for normal divisions in the basal cell layer?

4. The authors need to better characterise the shape of cells before and after divisions in their movies. This should be relatively easy. Ideally, they should determine:
i) Whether cells divide along their long cell axis? This would be expected to be the case.
ii) How division alters the shape of the clone: by comparing the shape of the mother cell measured just prior to division with the shape of the two daughter cells. Note it was previously argued based on work in suspended MDCK monolayers (Wyatt et al) that cell divisions relieve mechanical strain because they redistribute mass along the tension axis in a tissue in which there is no neighbour exchange. Does this hold for this tissue?
iii) How division alters cell height (comparing the height of mother cells and daughter cells). Note this shouldn't be maximum height as max height is usually determined by the size of the nucleus which is different between mother and daughter cells.
iv) How the mass of 1 cell, 2 cell, 3 cell and 4 cell clones compare.
v) How the mass and apical area of cells about to divide compare between cells that have yet to divide (1 cell clones) and cells that have already divided once (2 or 3 cell clones). This will show whether a size threshold determines the timing of division in this system as it does in many other systems. It will also help to address whether cells that lack the full complement of DNA grow.
vi) The model in Figure 5 makes an odd assumption about the scaling rules that apply, notably that height/mass/long axis change proportionally. The text suggests that this is a prediction. If it was a prediction I would like to know the underlying logic. If the assumption was made based on data, fine – say so.
vii) How do changes in organism size (HD, MD, LD) influence the shape and size of individual cells?

5. It is important to show how DNA content changes at division, not just say it is sub-G1. In the movies shown the nuclear marker appears to be inherited symmetrically. Are divisions symmetric? If this is the case, then the FACS profile should show that cells have either a 1n or 0.5n DNA content. Is this the case? If not, why not?

The authors suggest (line 187) that apical areas/cell volume/max height changes are expected to change with DNA content – based on observations in other systems. This makes little sense. A cell that doesn't have a full complement of its genome would not be expected to grow. Is this what is seen?
Note that max height likely measures nuclear height and is not a good measure of cell height.

6. The authors should explain what happens after 21 days? Are the cells born subsequent to this all diploid? Is the timing of this transition affected by Piezo perturbations?

Specific comments:
1. The term “asynthetic fission” is not a good term. The cells are still growing. It appears to be mitosis and cell division without DNA replication.
2. 162 “To determine whether the larval SECs are indeed in a post-mitotic state, devoid of DNA replication”. This is not the correct use of mitosis. Mitosis is DNA segregation and nuclear division not DNA replication.
3. In 3 cells clones is one cell always larger than the other two?

Referee #3 (Remarks to the Author):

In this study, the authors describe a fascinating new phenomenon of fission of skin cells during an extensive growth phase in zebrafish development. They developed tools to be able to follow skin cells over multiple cycles of these ‘asynthetic fission’ events and the imaging that is presented is stunning and the results are impressive. The study also does a great job in quantitively evaluating its findings and a model is presented that shows how asynthetic fission can lead to rapid expansion of surface area.
While overall the study and phenomenon is fascinating, some very major questions still stay open, one of the most fascinating ones being how the cells deal with what must be a randomly assorted genome that becomes smaller with each fission event. It is of course not possible to answer all upcoming questions in one study, but possibly some more explanations could be found in this area.
Another point is that the part about the tension sensing seems a bit underdeveloped compared to the other data presented in the study. Here more direct experiments could help to strengthen this point.
Otherwise, only minor questions about the data at hand still need to be clarified.

Major points

- as noted above, it is quite fascinating that these SEC cells can diminish their genome with each division. Do the authors see any apoptosis at all or do cells deal with whatever DNA content they

receive (as seen in Dzafic, Strzyz et al., Cell Reports 2015 during development)?
Further, from the data presented it is unclear how nuclear material is distributed between cells and whether each cell receives a similar amount of genetic material or if the distribution is random (at least roughly by overall content) between cells? By how much is the genome reduced typically in a cell after two division events and what is the minimal genome that is still sustainable? While the authors mention good arguments why these cells do not harm the organism as they have a short lifespan, it should also be commented why and how they survive at all. What if they lose housekeeping genes? How could this be prevented? This is a very fascinating question that could use some more insights.
Another interesting question along these lines that could be at least looked at in this study is how the division itself occurs: What does the spindle look like? How long does division take compared to 'normal' divisions'? Would this already indicate that checkpoint are skipped? Is this a typical cytokinesis? These observations would give important initial ideas to understand how genome depletion can still lead to functioning cells.

- The idea that cells get thinner after fission but increase their apical surface to cover more grounds is fascinating. However, what does this mean to the overall stratified epithelium? Is the loss in thickness of the SECs compensated by another cell layer? Does the fish get thinner at the same time? This could be further explained or explored.

- It is unclear why the complete set of data about the possible tension sensing ended up in the supplement. As mentioned above, this part of the study seems less well developed than the other parts. The rational for looking into stretch sensors and how they could act in this setting could be better explained. From the video and the montage in Figure S6a, the ablation measures seem a bit arbitrary at least for what is shown for 14dpf. Also, the drug treatments could be slightly better explored and maybe be backed up by genetic conditions. Is Piezo expressed in these cells at these stages? If yes where does it localize before and after growth induced stretch?

Minor points

- Figure 2b, c, could the authors explain why so much more variability is observed in standard length and trunk surface area at 10dpf than 8dpf?

- Figure 4a,b, BFP intensity is not necessarily a readout for a smaller genome as the genome could just be more tightly packed. However, 4g shows the differences more clearly so this data could move to the supplement if at all necessary.

- It would make it much easier for reviewers if the Figure number could be noted on the figure especially for the extended material, thank you.

Referee #4 (Remarks to the Author):

The authors investigate skin cell behavior in vivo with focus on cell expansion to achieve appropriate

body coverage. They have employed an innovative system of multicolor barcoding of cells combined with state-of-the-art quantitative imaging to study the dynamics of zebrafish skin development in vivo. The key new finding underlying the manuscript is that terminally-differentiated skin epithelial cell populations can increase in number through a process termed “asynthetic fission”, i.e. cell division without DNA duplication. Such divisions are well known in the meiotic division cycle as well as in some polyploid cell types (in particular liver cells), however, asynthetic fission is not established in relation to skin cell behavior.

Major points

1.) The authors suggest that in addition to the rapid supply of cells from stem- or progenitor cell populations, the differentiated cell population can also be “split” in these processes to rapidly support cell number demands. At this stage, it is unclear how relevant the described model will be across species and whether it reflects also the development of other non-keratinized stratified epithelium. Further, the authors do not analyze the molecular mechanisms underlying the fission process, which appears uncontrolled based on the current manuscript data. Obviously, uncontrolled fission is expected to be deleterious for cell fitness and function due to the unequal separation of the genome and cytoplasmic contents. The fission strategy is therefore counterintuitive, in fact, tissues of many organs (including epithelia) employ polyploidization as a strategy to cope with cell growth demands. In this regard, it is important for authors to ensure that SEC divisions take place in diploid cells rather than a subpopulation of polyploid cells.

2) The authors are largely focused on the organismal in vivo aspects of asynthetic fission. Unfortunately, they do not include in depth analysis at the cellular levels to advance our understanding of the highly interesting fission division process. Hence, the manuscript is descriptive and would benefit greatly from more careful analysis of SEC cells division. Particularly, the distribution of genomic material in the divided SEC cells should be addressed beyond the indirect assays applied in the manuscript. Flow cytometry analysis presented in figure 4g does not indicate clear haploid (1N) or hypo-haploid (0.5N) populations. This observation warrants further investigation to appreciate the distinction of the presented asynthetic fission form of cell division from mitotic (or meiotic) division. The presented systems should be amenable for deeper analysis by single cell sequencing. An alternative to single-cell sequencing could be karyotype analysis, preferably combined with multicolor FISH or similar.

3) Connected with point 2., it is also important for authors to characterize the division process in at least some detail since this process is the key discovery in the manuscript. Presumably, the dividing SEC cells go through stages of chromosome condensation, segregation, and cytokinesis. The authors should analyse fundamental characteristics of the mitotic division machinery such as centrosome, spindle, and kinetochore formation and behavior. Furthermore, basic questions along these lines have not been addressed. How does interference with chromosome segregation or cytokinesis impact the SEC population? Under these conditions respectively can mitotically trapped or binucleated SEC cells be identified?

4) Furthermore, the claim of DNA replication absence in SEC cells is not fully reflected in the data. It

appears in ED 4d that no replication was observed in SEC cells, however, this is using short EdU pulse. Notably, the replication block experiments with more long-term HU in fact indicate a very substantial role of DNA replication as seen in ED 4j-k (authors acknowledge this in very modest fashion stating “, the impact of HU treatment on SEC division was relatively moderate.”). Yet, this finding is not addressed, rather, authors focus very much on replication-independent fission divisions. Authors need to expand on this key point experimentally. This can be by evaluating DNA replication by other means than EdU, applying additional (non-HU) replication inhibitors, as well as applying additional time-series analysis.

5) In figure 4 f-h, the authors show a substantial increase in “subG1” SEC cells, however in this experiment it is unclear from which population of cells these are arising from as there is no clear loss of the G1 population. Continuous supply of “G1” SEC cells may account for this. Potentially, similar analysis of HU treated samples, which the authors indicate prevents BEC cell division but not SEC cells, could reveal this population and strengthen the presented observations.

6) Recently it was shown that tamoxifen binds to the sodium channel and impairs its recovery from the inactivated state, resulting in fewer channels available for reopening by subsequent depolarizations (https://doi.org/10.1016/j.molcel.2020.12.048). Given the suggested role of ion channels in the regulation of superficial epithelial cells (SECs) division, could tamoxifen treatment used for barcoding induction affect the observed process?

Minor points.
1. The approach presented in 4f-h largely excludes dying cells from the analysis however the terminology used “subG1” is commonly used to indicate an apoptotic cell population by flow cytometry. The authors should consider using a term such as sub-2N or sub-diploid in the figures and text to avoid confusion to the readers.
2. The presentation of FACS cell cycle profiles/DNA content is not optimal. In 4f, on the scatter plot authors gate (red square) for sub-2N (a much better term than sub-G1), but then authors show classical cell cycle histogram with 2N and 4N (and no sub-2N), with no explanation in figure legend.
3. In ED 4c, the EdU stain is seen in multiple samples and not always in the nucleus. What is the nature of the signal if not replication? At least in ED 4f, EdU signal was completely dependent on nucleotide supply as judged by the HU impact.

Author Rebuttals to Initial Comments:

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Point-by-point response

Referee #1:

The manuscript by Chan and coworkers presents a live imaging study of the superficial epidermal layer of the developing zebrafish. The authors show that the cells, previously considered postmitotic, undergo fission to generate cellular material for the expanding tissue. They further present data and modeling to suggest that the divisions occurs in the absence of DNA synthesis.

This is an intriguing manuscript that presents an interesting process of cell fission that could provide a rapid and effective means of tissue expansion. The imaging data is beautiful, of high quality, and convincing. The main shortcoming of the manuscript is that it describes the phenomenon of cell fission superficially and many of the quantitative data and statistical analyses do not appear robust. In particular the role of DNA synthesis is studied very superficially. Finally, while it is understandable that a full mechanism might not be fleshed out in a single study, the absence of any mechanistic analyses weakens the impact of the study.

Specific points:

1. The mean increase in SEC number in 10 dpf animals is in fact driven by 3 outlier animals, where the rest are in the range of the 8 dpf, which also likely explains why this result is not statistically significant and questions the robustness of this observation.

Response (1.1): We thank the referee for the encouraging comments on the study. We apologize for not clearly explaining the zebrafish rearing conditions. In our lab, we start feeding zebrafish larvae at 8 dpf, which usually leads to drastic yet variable growth by 10 dpf (Fig. 2b, c). Due to the natural variation in growth rates, the increased SEC number we observed at 10 dpf may seem less profound than might be expected (Fig. 2d). To address this issue, we further controlled zebrafish growth by manipulating the rearing density and showed that the SEC number increase is tightly correlated with the animal growth rate (Fig. 5i). We have revised the text to point out the high variation seen in Fig. 2d (page 6, paragraph 1) and commented on natural growth variation in the Methods (page 18, paragraph 1).

2. Given the data showing that unlabeled cells rapidly take over the tissue, as expected, hue balance should be quantified also at the 10 hpf time point and the data presented separately for each animal to exclude that clones arise from rare, leaky subrabasal or basal cells.

Response (1.2): As suggested, we performed total hue analyses at 8 and 10 dpf for each animal. We identified no significant difference in total hue numbers between these two time points. We have included this new result as Extended Data Fig. 2b.

To further exclude the possibility that rare labeled cells or clones may occasionally arise from suprabasal or basal cells, we retrospectively matched each 10 dpf SEC to its existence at 8 dpf in

the same animal. The match success rate was 100% (n = 396 cells from 4 animals), which strongly supports the idea that no cells or clones arise from suprabasal or basal cells during this two-day period. We have included this new result as Extended Data Fig. 3a, and updated the text on page 7, paragraph 1.

3. The data for decreased genome size is not robust as it could also be attributed to the shedding that occurs with a very high rate. The authors need to distinguish the subG1 cells that have divided versus the ones that are dying during shedding.

Response (1.3): We thank the referee for the comment. To determine whether genome size is indeed further reduced in divided SECs, we performed DAPI staining on fixed SECs to directly quantify their respective DNA content after each division. We are excited to report that the DAPI signals per cell in 1-cell, 2-cell, and 4-cell clones are proportionally reduced, consistent with our prior findings from histone level quantification (Fig. 4d, e) and flow-cytometry-based DNA content analyses (Fig. 4i-l). As divided SECs are readily retained on the animal body surfaces for at least two weeks (Extended Data Fig. 2), we suspect that the effect shown in Fig. 4l is not due to cell shedding that occurs at a very high rate. We have included the new DAPI data as Fig 4a-c, and updated the text on page 9, paragraph 2:

> "If a somatic cell population could divide without DNA replication, one would expect the progeny cells to have a reduced genome size. To examine this idea, we first determined relative changes in genomic DNA content by DAPI staining in fixed tissues. Remarkably, we found that nuclear DAPI signals per cell are reduced proportionally in 2-cell and 4-cell SEC clones (34% and 60% less, respectively; Fig. 4a, b), indicative of a sequential reduction in DNA content. Of note, we determined that the nuclear DAPI signals are specific and not affected by nuclear BFP2 expression, as tissue fixation eliminated the BFP2 signal in full (Fig. 4c)."

4. Describing the effect of HU on SEC clone size as "moderate" seems like a strong understatement. The data suggest that both the clone size distribution and the divided pool are restored to near 9hpf levels. It is also not clear why a student´s t-test is used for statistical testing of Ext. Data Fig. 4k (divided cell pool) as the authors are comparing 3 groups, so a t-test is inappropriate and a Anova-type test should be used. Also, a post hoc test to separately compare the difference between untreated and treated at 10hpf is required, as this most likely would show a significant, rather than a moderate effect of the treatment.

Response (1.4): We appreciate the referee's comment and revised the section as suggested. We have now applied one-way ANOVA to the results of Extended Data Fig. 5k, and we reported p-values from post hoc tests for each examined group. In addition, we revised the text regarding the HU effect on SEC division on page 8, paragraph 2:

> "Despite its elimination of nearly all EdU-positive BECs (~97%; Extended Data Fig. 5e-g), the HU treatment failed to block SEC division to a similar extent."

5. The authors need to scrutinize statistical testing throughout the manuscript and apply appropriate anova-type tests instead of t-tests when 3 or more groups are being compared.

Response (1.5): As suggested, we have checked all statistical tests throughout the manuscript. We replaced t-tests with one-way ANOVA when 3 or more groups are compared. We have updated the statistical information for each of the affected figures and figure legends (Fig. 4b, e, f, g, h; Fig. 5e, f, h, i, k, l, m and Extended Data Fig. 5k).

6. Although in the abstract the authors state that the process occurs "presumably through tension-mediated activation of stretch-activated ion channels", no mechanistic analyses are provided and no data for ion channel involvement is presented.

Response (1.6): We apologize for not making this part clear. The analyses and the data for ion channel data involvement were shown in the supplemental figures (Extended Data Fig. 6 in the previous version). In this revised manuscript, we are excited to report that we have added new results to support the expression of Pizeo1 in the SEC population, and we have genetically tested the involvement of Piezo1 in SEC division. We have included these new results as Fig. 6j-r and Extended Data Fig. 9g, h, and revised the text on page 15, paragraph 1:

> "To determine whether Pizeo1 is indeed required for asynthetic fission, we applied a CRISPR/Cas9-based approach to disrupt Piezo1 expression in live zebrafish larvae. Injection of Piezo1 sgRNAs at the 1-cell stage effectively reduced Piezo 1 expression in vivo (decrease of 53%; Fig. 6j-l), leading to significant suppression of the SEC division event in 8 dpf palmskin (39% less; Fig. 6m-p). Of note, we detected no growth abnormality in the sgRNAs injected animals, in terms of body length or body surface area (Fig. 6q, r). We further determined that Piezo1 is present as cytoplasmic puncta in SECs regardless of growth-induced stretch (Extended Data Fig. 9g, h). Taken together, the data lead us to conclude that the activity of stretch-activated ion channels, presumably including Piezo1, is key to stimulation of asynthetic fission."

7. The last sentence of the abstract: "This frugal yet flexible mode of cell proliferation may also be relevant to non-skin cells and disease conditions" is very speculative at this point and would be better removed.

Response (1.7): We appreciate the referee's comment and respectfully request the referee will allow us to speculate on the relevance of our findings, which in our view describe a variant form of cell proliferation event in a vertebrate tissue. We have revised the last sentence of the abstract as follows: "This frugal yet flexible mode of cell proliferation might also occur in contexts other than zebrafish skin expansion."

Minor

1. It is not clear how the SEC Coverage ratio (Fig 2g) is defined and computed.

Response (1.8): We have revised the text in the Methods section on page 25, paragraph 1:

"To determine the SEC coverage ratio, the area occupied by the recombined cells and the entire trunk area (stitched images) were analyzed using thresholding methods in FIJI."

Referee #2:

This paper by Chen and co-authors reports a fascinating new discovery. Using clonal labelling methods to mark individual outer skin cells of the zebrafish larvae they report rounds of cell division that occur in the absence of DNA replication. During a specific phase in the animals life (prior to day 21), this leads to the formation of small clones of cells in the skin with reduced DNA content. By imaging these divisions live they show that cell division is accompanied by DNA segregation, making it appear as if this is a case of cells entering mitosis without undergoing DNA replication (as occurs in fission yeast cut mutants).

Since these division events are more prevalent in larger animals, which have more surface to cover, and correlate with local differences in surface growth, they suggest that these likely contribute to the ability of the skin to cover the growing embryo. Finally, they show that these are dependent on Piezo channels using gain and loss of function drug perturbations.

The paper is well written, includes a large number of relevant experiments that are well-controlled and quantified appropriately and lots of stunning images. It promises to open up a whole new area of biology, will interest a wide audience, and would therefore be appropriate for a journal like Nature.

Before being published however there are a number of issues that should be addressed. Most could be addressed by alterations to the text, additional analyses, and a small number of experiments.

Key concerns:

1. Interestingly, previous work in Drosophila larvae (following wounding) and C. elegans (development) suggests that skin cells fuse to ensure good coverage of the animal. Therefore more needs to be done to make it clear how this type of division aids coverage of the animal with limited material as the authors suggest. In fact, by undergoing division cells need more plasma membrane not less – something might raise tension.

Response (2.1): First, we thank the referee for the encouraging comments on our study. Of note, we also identified cell fusion in the zebrafish skin, but it occurs only at a very low frequency (Extended Data Fig. 4). Why this distinct type of cell division is more favored over cell fusion is indeed a fascinating question. We speculate that this division mode may be unique to the stratified epithelium (the skin tissues of both fly and c. elegans are monolayers) or it could be a new invention in vertebrate tissues. However, we would like to highlight a key assumption in the geometric model we proposed, which can predict the observed morphological changes of divided SECs, i.e., no additional synthesis of cytosol content would occur during sequential divisions. Thus, we propose that "asynthetic fission" is economically more efficient than cell fusion for producing more apical surfaces

on a large scale. We have revised the text to clearly state key assumptions we made about the asynthetic fission on page 11, paragraph 2.

> "Thus, we presumed that during SEC division: (1) there is no need to synthesize new materials; (2) only existing cellular components, including plasma membrane and cytoplasm, are utilized to construct the new cells after each division."

2. The data on Gd+++ and Yoda are exciting and should be in the main text. They imply that division is dependent on Piezo. Although not essential, it would be good to show this with a genetic experiment. In addition, it would be good to determine the consequences of these treatments for the ability of the animal to recover from wounding.

Response (2.2): As suggested, we have now included the Gd3+ and Yoda data as a main figure (new Fig. 6). In addition, we have genetically tested the involvement of Piezo 1. Using a CRISPR/Cas9-based approach, we determined that when the Piezo1 expression level is reduced in a mosaic pattern, fewer SECs can proceed to division. This finding suggests that Piezo1 is required for the division, consistent with the results from the Gd3+ and Yoda1 treatment experiments. We have included these new results as Fig. 6j-r, and updated the text on page 15, paragraph 1:

> "To determine whether Pizeo1 is indeed required for asynthetic fission, we applied a CRISPR/Cas9-based approach to disrupt Piezo1 expression in live zebrafish larvae. Injection of Piezo1 sgRNAs at the 1-cell stage effectively reduced Piezo 1 expression in vivo (decrease of 53%; Fig. 6j-l), leading to significant suppression of the SEC division event in 8 dpf palmskin (39% less; Fig. 6m-p). Of note, we detected no growth abnormality in the sgRNAs injected animals, in terms of body length or body surface area (Fig. 6q, r). We further determined that Piezo1 is present as cytoplasmic puncta in SECs regardless of growth-induced stretch (Extended Data Fig. 9g, h). Taken together, the data lead us to conclude that the activity of stretch-activated ion channels, presumably including Piezo1, is key to stimulation of asynthetic fission.

Response (2.3): As suggested, we tested the effect of Gd3+ and Yoda treatments on SEC division after wounding. However, we found that neither treatment has a significant influence on the extent of SEC division, which still occurs at a high rate in the wounded tissues. We suspect that Piezo 1 may either be inactivated or remain over-activated upon wounding. Thus, the treatments and the cell tracking schemes may need to be further optimized. We hope the referee to allow us to omit these inconclusive results.

In doing so the authors should test:

i) The apical area and height of cells in Gd+++, control and Yoda treated animals.

Response (2.4): Because the Gd3+ and Yoda treatments are most effective to affect SEC division during growth, we determined the "apical surface area" and "median cell height" in Gd3+ and Yoda treated animals at 10 dpf. We have now included the quantitative data in Extended Data Fig. 9a and 9b, and we commented on the findings on page 14, paragraph 1 (see Response 2.8).

*ii) The orientation of **the long cell axis** in all conditions.*

Response (2.5): As suggested, we determined "the long cell axis" in all conditions during growth. We have now included the new quantitative data in Extended Data Fig. 9c, and we commented on the findings on page 14, paragraph 1 (see Response 2.8).

iii) DNA content in all conditions. Are cells diploid in Gd+++ treated cells?

Response (2.6): As suggested, we determined the "binucleated SEC ratio" in all conditions during growth. We have now included the new quantitative data in Extended Data Fig. 9f, and we commented on the findings on page 14, paragraph 1 (see Response 2.8).

iv) How do these treatments alter the loss of these SEC cells as animals mature?

Response (2.7): As suggested, we determined the extent of the SEC loss as animals mature in all conditions. We have now included the new quantitative data in Extended Data Fig. 9e, and we commented on the findings on page 14, paragraph 1 (see Response 2.8).

v) Why do there appear to be lots of dark clones with large number of small cells in Gd images (S6)? Is this taken into account when assessing Trunk surface area?

Response (2.8): We determined that both Gd3+ and Yoda1 treatments affect SEC coverage at 10 dpf. We have included the new quantitative data in Extended Data Fig. 9e, and we commented on the findings on page 14, paragraph 1. Also, we assessed "Trunk surface area" using bright-field images that were independently captured. Thus, the quantification is not affected by dark areas seen in the images. We have updated the Methods section for clarification (page 19, paragraph 1).

> "We further determined that the treatments differentially affected apical surface area, median cell height, the long cell axis, and the SEC coverage at later stages, but had no effects on the ratio of binucleated cells (Extended Data Fig. 9a-f). Notably, Yoda1 treatment had no measurable effects on fish body length and body surface area (Fig. 6h, i), supporting the idea that the asynthetic fission phenotypes are unlikely to be caused directly by growth perturbations."
>
> "For measuring Standard length and Trunk surface area, bright-field images were independently captured using a Leica M205 stereo microscope."

3. The authors should do more to test the extent to which the cells undergo a conventional mitosis before they divide. Thus, far their paper only includes a few low resolution images which suggest that nuclear division precedes cell division.

i) To do so, they should image spindles (can be done in fixed tissues), DNA (with DAPI/Hoechst or equivalent) to measure DNA content using microscopy, and Lamin or equivalent to see if the nuclear compartment boundary is lost at division. It would be great (but not essential) to know if CDK inhibitors arrest the process as would be expected.

Response (2.9): We thank the referee for providing these insightful suggestions that, in our view, significantly expand the scope of our findings. As suggested, we performed immunostaining on whole-mount fixed palmskin animals with antibodies against alpha-tubulin (mitotic spindles) and gamma-tubulin (centrosomes). Remarkably, we identified "tripolar spindles" in many dividing SECs (20%; n = 26/128), which provides direct evidence that DNA segregation during SEC division is error-prone. In sharp contrast, we found no such spindle abnormalities in any of the dividing BECs (0%; n = 0/108), i.e., the skin stem cell population. We have included these important new results as Fig. 4q and updated the text on page 10, paragraph 2:

> "Notably, immunostaining of mitotic spindles and centrosomes further revealed many dividing SECs with "tripolar spindles". We saw no such spindle abnormalities in any of the dividing BECs (0% in BECs vs. 20% in SECs; n = 0/108 and 26/128 cells, respectively; Fig. 4q)."

Response (2.10): As suggested, we performed DAPI staining on whole-mount fixed palmskin animals to determine DNA content in not-yet-divided and divided SECs. We are excited to report that the DAPI signals per cell in 1-cell, 2-cell, and 4-cell clones were reduced proportionally, consistent with our findings from the histone level quantification and the flow-cytometry-based DNA content analyses. We have included the new results as Fig 4a-c, and updated the text on page 9, paragraph 2:

> "If a somatic cell population could divide without DNA replication, one would expect the progeny cells to have a reduced genome size. To examine this idea, we first determined relative changes in genomic DNA content by DAPI staining in fixed tissues. Remarkably, we found that nuclear DAPI signals per cell were reduced proportionally in 2-cell and 4-cell SEC clones (34% and 60% less, respectively; Fig. 4a, b), indicative of a sequential reduction in DNA content. Of note, we determined that the nuclear DAPI signals are specific and not affected by nuclear BFP2 expression, as tissue fixation eliminated the BFP2 signal in full (Fig. 4c)."

Response (2.11): As suggested, we conducted a small-scale chemical screen to determine whether inhibitors of CDKs and other cell cycle components may influence the process. In brief, we treated palmskin animals with HU, RO3306, Paclitaxel, Roscovitine, and Nocodazole for 12 hours at 9.5 dpf. Intriguingly, we found that SEC division is only most sensitive to the tubulin polymerization inhibitor Nocodazole. The treatment causes a significant reduction in the divided pool and a marked increase in the binucleated cells. We have now included these new results as Extended Data Fig. 6, updated the Methods section, and revised the text on page 10, paragraph 2:

> "To determine whether common cell cycle machineries may mediate asynthetic fission, we tested the effects of a panel of cell cycle inhibitors. Intriguingly, we determined that the division process is only highly sensitive to the treatment of the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6)."

ii) If cells assemble a spindle prior to division this could guide the orientation of the division axis.

Response (2.12): Yes. We have now included the spindle immunostaining data as Fig. 4q.

iii) It is not clear how mitosis can proceed normally if chromosomes are not able to form bipolar attachments. Is mitosis as a result slower in these divisions than it is for normal divisions in the basal cell layer?

Response (2.13): This is an interesting question. As suggested, we determined the length of the mitotic phase in both the SEC and the basal BEC population. To enable a direct comparison of both cell division events, we created an additional new transgenic line Tg(krt4:H2A-EGFP) to monitor the SEC nuclear division process. We then generated a double transgenic animal, Tg(krt19:H2A-mCherry; krt4:H2A-EGFP), for live monitoring of both SEC and BEC divisions as they occur simultaneously in the same animal. Intriguingly, just as the referee predicted, we determined that the period from prophase to telophase is markedly lengthened in the SEC population (70.2 ± 18.22 min in BECs vs 108.3 ± 26 min in SECs; n = 97 captured events). Surprisingly, in these time-lapse images, we also happened to observe a collection of the chromosome segregation defects, such as “anaphase bridges”, “lagging chromosomes”, “micronuclei”, and “tripolar segregation” in the dividing SECs. These live imaging results provide further evidence that SEC division does not proceed as typical mitosis or meiosis; instead, it is evidently highly error-prone. We have included these important new results as Fig. 4m-p and Supplementary Video 8 and 9. We also deposited the new code that we generated for analysis (page 28), and updated the Methods section (page 27) and the text on page 10, paragraph 2:

> “To monitor the nuclear division process in SECs in real time, we created a transgenic line, Tg(krt4:H2A-EGFP), to capture chromosome segregation dynamics during SEC division. Remarkably, we identified frequent chromosome segregation defects in the dividing SECs but not BECs, including “anaphase bridges”, “lagging chromosomes”, “micronuclei”, and “tripolar segregation” (Fig. 4m, n; Supplementary Video 8, 9). Time-lapse imaging of double transgenic animals further showed that the division process is markedly lengthened in SECs, as determined by the period from prophase to telophase (70.2 ± 18.22 min in BECs vs 108.3 ± 26 min in SECs; n = 97 captured events; Fig. 4m, o, p; Methods).”

4. The authors need to better characterise the shape of cells before and after divisions in their movies. This should be relatively easy. Ideally, they should determine:

i) Whether cells divide along their long cell axis? This would be expected to be the case.

Response (2.14): As suggested, we determined the long cell axis before and during the division. Intriguingly, SECs indeed divide along their long cell axis as the referee has predicted. We have included the new quantitative data in Extended Data Fig. 3b.

ii) How division alters the shape of the clone: by comparing the shape of the mother cell measured just prior to division with the shape of the two daughter cells. Note it was previously argued based on work in suspended MDCK monolayers (Wyatt et al) that cell divisions relieve mechanical strain

because they redistribute mass along the tension axis in a tissue in which there is no neighbour exchange. Does this hold for this tissue?

Response (2.15): As suggested, we determined cell shape changes in the mother and the daughter cells. Intriguingly, we found that the daughter cells are more rounded as determined by two shape factors (i.e. aspect ratio and circularity). These findings, together with the results mentioned in Response (2.14), are supportive of the model proposed by Wyatt et al. We have now included the quantitative data in Extended Data Fig. 3c and 3d, added the reference, and updated the text on page 7, paragraph 1:

> "At the cellular level, division was biased along the long cell axis. Divided cells were also evidently more rounded, as determined by aspect ratio and circularity (Extended Data Fig. 3b-d), which is suggestive of stress relaxation[16]."

iii) How division alters cell height (comparing the height of mother cells and daughter cells). Note this shouldn't be maximum height as max height is usually determined by the size of the nucleus which is different between mother and daughter cells.

Response (2.16): As suggested, we measured changes in cell height from the movies that capture 1-to-4 division trajectories. We determined "median cell height", which was computed from the z-directional heights of a cell. We determined that the "median cell height" is reduced upon the division, consistent with the result from the measurement of maximum cell height (Fig. 4g). We have now included the new data in Extended Data Fig. 7h and 9b. We also updated the Methods on page 26, paragraph 2, deposited the new code that we generated for analysis (page 28), and revised the text on page 11, paragraph 2.

iv) How the mass of 1 cell, 2 cell, 3 cell and 4 cell clones compare.

Response (2.17): As suggested, we measured changes in cell volumes from the movies that capture 1-to-4 division trajectories. We found that although the individual cell volume is reduced, the combined volume of divided cells is relatively constant. This finding is consistent with the result from the analyses of snapshot images (Fig. 4h). We have now included the new data in Extended Data Fig. 7a-g, and updated the text on page 11, paragraph 2.

v) How the mass and apical area of cells about to divide compare between cells that have yet to divide (1 cell clones) and cells that have already divided once (2 or 3 cell clones). This will show whether a size threshold determines the timing of division in this system as it does in many other systems. It will also help to address whether cells that lack the full complement of DNA grow.

Response (2.18): As suggested, we determined both the cell volume and apical surface area from SECs that are about to divide for the first time vs cells that are about to divide the second time. We found that the division is not gated by a specific size threshold, as smaller cells (i.e. cells that have divided one time) can also readily divide. We have now included the new quantifications as Extended Data Fig. 7i-k, and updated the text on page 11, paragraph 2.

vi) The model in Figure 5 makes an odd assumption about the scaling rules that apply, notably that height/mass/long axis change proportionally. The text suggests that this is a prediction. If it was a prediction I would like to know the underlying logic. If the assumption was made based on data, fine – say so.

Response (2.19): This is an interesting question. We are happy to explain our rationale here. We determined that (1) the cells have no DNA replication activity, and (2) the DNA content is reduced upon each division; furthermore, (3) DNA content is known to correlate with cell size in general. We thus hypothesized that the cells may simply re-distribute whatever constituents are present upon each division. Given that we have no idea how these divided cells may look, we generated the model based on the scaling law, which in our view is the simplest and requires the fewest assumptions. For clarification, we have revised the text on page 11, paragraph 2:

> "As SECs underwent subsequent divisions, we found that individual cells exhibit drastic yet proportional reductions in cell height and volume (Fig. 4g, h). By examining the individual cells throughout a 1-to-4-cell division trajectory, we determined that the division is not gated by a size threshold; the total combined cell volume appears to be relatively constant (n = 5 clones; Extended Data Fig. 7). Thus, we presumed that during SEC division: (1) there is no need to synthesize new materials; (2) only existing cellular components, including plasma membrane and cytoplasm, are utilized to construct the new cells after each division."

vii) How do changes in organism size (HD, MD, LD) influence the shape and size of individual cells?

Response (2.20): As suggested, we determined how changes in organism size (HD, MD, LD) may affect the shape and size of individual cells. For the size, we determined that the apical surface area on average is markedly reduced under the LD condition. In terms of shape, we detected slight changes when comparing the HD with the LD condition. We have now included these new results as Fig. 5l and 5m, and updated the text on page 13, paragraph 1.

5. It is important to show how DNA content changes at division, not just say it is sub-G1. In the movies shown the nuclear marker appears to be inherited symmetrically. Are divisions symmetric? If this is the case, then the FACS profile should show that cells have either a 1n or 0.5n DNA content. Is this the case? If not, why not?

Response (2.21): Yes. We fully agree that it is important to show how DNA content changes at division. As mentioned above (2.10), we have now performed DAPI staining to determine changes in DNA content upon each division and found that the DAPI signal per cell is proportionally reduced in 2- and 4-cell clones. We have included these new results in Fig. 4a-c.

Response (2.22): As mentioned above (2.9 and 2.13), we monitored SEC divisions and observed "anaphase bridges" and "lagging chromosomes", "micronuclei", and "tripolar segregation" activities. As these findings provide direct support that SEC division at DNA level is not well regulated as in mitosis or meiosis, we speculate that the division is likely to be imprecise or non-symmetric, which could explain why the FACS profile does not include sharp peaks representing cells that have either

a 1N or 0.5N DNA content. For clarification, we have now commented on this point on page 11, paragraph 1:

> "… we conclude that unlike mitosis or meiosis, asynthetic fission is error-prone, with a putative absence of well-orchestrated chromosome alignment during the division process."

The authors suggest (line 187) that apical areas/cell volume/max height changes are expected to change with DNA content – based on observations in other systems. This makes little sense. A cell that doesn't have a full complement of its genome would not be expected to grow. Is this what is seen?

Response (2.23): We thank the reviewer for the comment and would like to explain our view on this matter. Although individual SECs may appear to be growing during the divisions in the movie (a 2-demensional view), our quantifications of cell volume (in 3-demensional space) and the model suggest that there is little or no growth upon each division. We further found that the total combined volumes are constant before and after the division (1-to-4-cell division trajectories were examined from the movies; Extended Data Fig. 7). We have included these new results in Extended Data Fig. 7a-g, and revised the text on page 11, paragraph 2.

Note that max height likely measures nuclear height and is not a good measure of cell height.

Response (2.24): We have now determined the "median cell height" as mentioned above (2.16), which was computed based on z-directional heights of a cell. The new data are included in Extended Data Fig. 7h and 9b. The methods were updated on page 26, paragraph 2. The new code we generated for analysis was deposited (page 28).

6. The authors should explain what happens after 21 days? Are the cells born subsequent to this all diploid? Is the timing of this transition affected by Piezo perturbations?

Response (2.25): Yes, the "sub-2N" population is greatly reduced at later and adult stages according to flow-cytometry-based DNA content analyses (Fig. 4l). We have commented on this point on page 10, paragraph 1. Also, we have determined that the Piezo1 perturbations can affect the transition (Response 2.7). We have included the results in Extended Data Fig. 9d and 9e, and updated the text on page 14, paragraph 1.

Specific comments:

1. The term "asynthetic fission" is not a good term. The cells are still growing. It appears to be mitosis and cell division without DNA replication.

Response (2.26): Based on the cell size quantifications and the geometric model, we conclude that SECs are not growing during division. The expansion in "apical surface area" after the division comes at the expense of the cell height and to some extent, volume. Thus, SECs are smaller and thinner after each division. Our observations suggest that (1) the total combined cell volumes are constant before and after the division (no net increase in cellular volumes); (2) the process has no detectable DNA synthesis; (3) it is not a typical type of mitosis nor meiosis (i.e. DNA segregation defects are

prevalent; Fig. 4m-q). Thus, we respectfully suggest that “asynthetic fission” may be a proper term for this division process with as yet unknown regulatory features.

2. 162 “To determine whether the larval SECs are indeed in a post-mitotic state, devoid of DNA replication”. This is not the correct use of mitosis. Mitosis is DNA segregation and nuclear division not DNA replication.

Response (2.27): As suggested, we have removed the statement on page 8, paragraph 2.

3. In 3 cells clones is one cell always larger than the other two?

Response (2.28): This question is intriguing. Based on the geometric model we proposed, one would indeed predict that in 3-cell clones, one cell shall always be larger than the other two because the largest one is yet to divide. Here, we performed “apical surface area” and “cell volume” analyses on the same cell as it divides into a 4-cell clone. We are excited to report that in all cases, one cell was always larger than the other two in a 3-cell clone, consistent with what the model would have predicted. We have included this new result in Extended Data Fig. 7l and 7m.

Referee #3:

In this study, the authors describe a fascinating new phenomenon of fission of skin cells during an extensive growth phase in zebrafish development. They developed tools to be able to follow skin cells over multiple cycles of these ‘asynthetic fission’ events and the imaging that is presented is stunning and the results are impressive. The study also does a great job in quantitively evaluating its findings and a model is presented that shows how asynthetic fission can lead to rapid expansion of surface area.

While overall the study and phenomenon is fascinating, some very major questions still stay open, one of the most fascinating ones being how the cells deal with what must be a randomly assorted genome that becomes smaller with each fission event. It is of course not possible to answer all upcoming questions in one study, but possibly some more explanations could be found in this area.

Another point is that the part about the tension sensing seems a bit underdeveloped compared to the other data presented in the study. Here more direct experiments could help to strengthen this point.

Otherwise, only minor questions about the data at hand still need to be clarified.

Major points

- as noted above, it is quite fascinating that these SEC cells can diminish their genome with each division. Do the authors see any apoptosis at all or do cells deal with whatever DNA content they receive (as seen in Dzafic, Strzyz et al., Cell Reports 2015 during development)?

Response (3.1): First, we thank the reviewer for the encouraging comments on our study. To determine whether apoptosis may be activated during SEC division, we created a new transgenic reporter line, Tg(ubi:H2A-mCherry-2A-GC3AI), to monitor caspase-3 activation in the SEC population. We determined that the reporter is functional, as a pro-apoptotic UV stimulus readily activates the reporter in SECs. Using the reporter line, we did not detect any apoptotic cells in the SEC population at 8 and 10 dpf (n = 27 animals), consistent with a prior zebrafish study (Slanchev et al., Plos Genetics). We have included this new result in Extended Data Fig. 10 and revised the text on page 16, paragraph 1.

Further, from the data presented it is unclear how nuclear material is distributed between cells and whether each cell receives a similar amount of genetic material or if the distribution is random (at least roughly by overall content) between cells? By how much is the genome reduced typically in a cell after two division events and what is the minimal genome that is still sustainable? While the authors mention good arguments why these cells do not harm the organism as they have a short lifespan, it should also be commented why and how they survive at all. What if they lose housekeeping genes? How could this be prevented? This is a very fascinating question that could use some more insights.

Response (3.2): In the revised version, we have included data from our experiment in which DAPI staining was performed to directly measure DNA content in the divided SECs. We determined that the DAPI signals per cell in 2- and 4-cell clones are proportionally reduced, consistent with our prior findings from the histone level quantification and the flow-cytometry-based DNA content analyses. We have included the new results in Fig.4a-c, and described the findings on page 9, paragraph 2:

> "If a somatic cell population could divide without DNA replication, one would expect the progeny cells to have a reduced genome size. To examine this idea, we first determined relative changes in genomic DNA content by DAPI staining in fixed tissues. Remarkably, we found that nuclear DAPI signals per cell were reduced proportionally in 2-cell and 4-cell SEC clones (34% and 60% less, respectively; Fig. 4a, b), indicative of a sequential reduction in DNA content. Of note, we determined that the nuclear DAPI signals are specific and not affected by nuclear BFP2 expression, as tissue fixation eliminated the BFP2 signal in full (Fig. 4c)."

Response (3.3): Why and how do these cells survive is indeed a fascinating question. We speculate that the role of nuclei may be negligible for certain terminally differentiated cells. For instance, mammalian red blood cells do not have nuclei but remain functional in vivo for as long as 120 days. By comparison, the idea that SECs with reduced genome size can live up to 2 weeks may seem less extreme. We thank the reviewer for these intriguing questions and have now commented on this point in the Discussion section on page 17, paragraph 1:

> "Future studies will be needed to determine how SECs are able to bypass cell cycle checkpoints and partition non-duplicated genomic DNA into daughter cells. It will also be interesting to ask whether meiotic machineries, Piezo1 dynamics, or Hippo/YAP signaling may be involved in these processes. Another open question is how SECs would be able to keep up with basic

physiological needs without an intact genome, a situation reminiscent of anucleated erythrocytes in mammals."

Another interesting question along these lines that could be at least looked at in this study is how the division itself occurs: What does the spindle look like? How long does division take compared to 'normal' divisions'? Would this already indicate that checkpoint are skipped? Is this a typical cytokinesis? These observations would give important initial ideas to understand how genome depletion can still lead to functioning cells.

Response (3.4): We thank the referee for these key questions. As suggested (also suggested by referee #2), we performed immunostaining on whole-mount fixed *Tg(krt4:H2A-EGFP)* animals with antibodies against alpha-tubulin (mitotic spindles) and gamma-tubulin (centrosomes). Remarkably, we identified "tripolar spindles" in many dividing SECs (20%; n = 26/128), which provides direct evidence that DNA segregation during SEC division is error-prone. In sharp contrast, we found no such spindle abnormalities in any dividing BECs (0%; n = 0/108), i.e., the skin stem cell population. We have included these important new results in Fig. 4q and updated the text on page 10, paragraph 2:

"Notably, direct immunostaining targeting the structures of mitotic spindles and centrosomes further revealed many dividing SECs with "tripolar spindles". We saw no such spindle abnormalities in any of the dividing BECs (0% in BECs vs. 20% in SECs; n = 0/108 and 26/128 cells, respectively; Fig. 4q)."

Response (3.5): As suggested (also suggested by referee #2), we determined the length of the mitotic phase in both the SEC and the basal BEC population. To enable a direct comparison of both cell division events, we generated a double transgenic animal, Tg(krt19:H2A-mCherry; krt4:H2A-EGFP), for live monitoring of both SEC and BEC divisions as they occur simultaneously in the same animal. Intriguingly, we found that the period from prophase to telophase is significantly lengthened in the SEC population (70.2 ± 18.22 min in BECs vs 108.3 ± 26 min in SECs; n = 97 captured events). Surprisingly, from the time-lapse images, we happened to capture a collection of the chromosomal segregation defects, such as "anaphase bridges", "lagging chromosomes", "micronuclei", and "tripolar segregation" in the dividing SECs. These live imaging results provide further evidence that SEC division does not proceed as typical mitosis or meiosis; instead, it is evidently error-prone. We have included these important new results as Fig. 4m-p, and Supplementary Video 8 and 9, updated the Methods section and the text on page 10, paragraph 2:

"To monitor the nuclear division process in SECs in real time, we created a transgenic line, Tg(krt4:H2A-EGFP), to capture chromosome segregation dynamics during SEC division. Remarkably, we identified frequent chromosome segregation defects in the dividing SECs but not in the BECs, including "anaphase bridges", "lagging chromosomes", "micronuclei", and "tripolar segregation" (Fig. 4m, n; Supplementary Video 8, 9). Time-lapse imaging of double transgenic animals further showed that the division process is markedly lengthened in SECs,

as determined by the period from prophase to telophase (70.2 ± 18.22 min in BECs vs 108.3 ± 26 min in SECs; n = 97 captured events; Fig. 4m, o, p; Methods)."

- The idea that cells get thinner after fission but increase their apical surface to cover more grounds is fascinating. However, what does this mean to the overall stratified epithelium? Is the loss in thickness of the SECs compensated by another cell layer? Does the fish get thinner at the same time? This could be further explained or explored.

Response (3.6): We thank the reviewer for this very interesting question and would like to provide our view. During the developmental time window we studied, zebrafish larvae skins are in the midst of transitioning from a bi-layered structure to a multi-layered epidermis. We speculate that "asynthetic fission" is a temporal, backup mechanism for supporting body coverage prior to the formation of the additional suprabasal layer at later developmental stages. Thus, despite the fact that individual SEC cells are thinner upon each division, the skin epidermis as a whole is in the process of becoming thicker during this time period (Guzman et al., Biol Open). We have now included a relevant reference and explanation in the Discussion section on page 16, paragraph 2:

"Through asynthetic fission, skin cells devoid of mitotic potential may retain a means of producing more apical surface area as the larval skin gradually transitions from a bi-layered structure to a multi-layered epidermis[13]. As SECs last only a few weeks on the body surface and are eventually all shed during development, these hypoploid cells are not likely to cause harm to the individual."

- It is unclear why the complete set of data about the possible tension sensing ended up in the supplement. As mentioned above, this part of the study seems less well developed than the other parts. The rational for looking into stretch sensors and how they could act in this setting could be better explained. From the video and the montage in Figure S6a, the ablation measures seem a bit arbitrary at least for what is shown for 14dpf. Also, the drug treatments could be slightly better explored and maybe be backed up by genetic conditions. Is Piezo expressed in these cells at these stages? If yes where does it localize before and after growth induced stretch?

Response (3.7): As suggested (also by the referee #2), we now include the entire set of results related to tension sensing in the main figures (new Fig. 6). In addition, we followed the advice to further expand our exploration of the mechanism by testing the consequences of the Gd3+ and Yoda1 treatments on cell apical surface area, cell height, long cell axis, SEC coverage and the binucleated cell ratio, on animal body surfaces (Extended Data Fig.9). Moreover, we have genetically tested the involvement of Piezo 1. Through a CRISPR/Cas9-based approach, we determined that when the Piezo1 expression level is reduced in a mosaic pattern, fewer SECs can proceed to division. This finding suggests that Piezo1 is required for the division and is consistent with the results from the Gd3+ and Yoda1 treatments. We have included these new results in Fig. 6j-r, and updated the text on page 15, paragraph 1:

> "To determine whether Pizeo1 is indeed required for asynthetic fission, we applied a CRISPR/Cas9-based approach to disrupt Piezo1 expression in live zebrafish larvae. Injection of Piezo1 sgRNAs at the 1-cell stage effectively reduced Piezo 1 expression in vivo (decrease of 53%; Fig. 6j-l), leading to significant suppression of the SEC division event in 8 dpf palmskin (39% less; Fig. 6m-p). Of note, we detected no growth abnormality in the sgRNAs injected animals, in terms of body length or body surface area (Fig. 6q, r).

Response (3.8): To determine Piezo1 dynamics in SECs, we performed immunostaining for Piezo1. We first examined the specificity of the Ab by testing it on the Piezo1 sgRNA-injected animals. We then determined that Piezo1 localization in SECs shows no marked difference at 8 and 14 dpf (less stretched vs. more stretched tissues). We have included these new results as Extended Data Fig. 9g, h, and updated the text on page 15, paragraph 1:

> "We further determined that Piezo1 is present as cytoplasmic puncta in SECs regardless of growth-induced stretch (Extended Data Fig. 9g, h). Taken together, the data lead us to conclude that the activity of the stretch-activated ion channels, presumably including Piezo1, is key to stimulation of asynthetic fission."

Minor points

- Figure 2b, c, could the authors explain why so much more variability is observed in standard length and trunk surface area at 10dpf than 8dpf?

Response (3.9): We thank the reviewer for pointing out this aspect of the data. In the lab, we start feeding zebrafish larvae at 8 dpf, which usually leads to a drastic yet variable growth by 10 dpf (Fig. 2d, c). We have revised the text to alert the reader to the variation seen in Fig. 2d (page 6, paragraph 1) and commented on the natural growth variation in the Methods (page 18, paragraph 1).

- Figure 4a,b, BFP intensity is not necessarily a readout for a smaller genome as the genome could just be more tightly packed. However, 4g shows the differences more clearly so this data could move to the supplement if at all necessary.

Response (3.10): In Fig. 4a, b (now new Fig. 4d, e), we determined the integrated intensity of BFP2 in each divided SEC as the sum of pixel values in the image. We reasoned that the fluorescence signal readouts are quantitative and not greatly affected by whether regions of interest are condensed or not, as long as the recorded pixels are not saturated. We have now revised the text in the figure legend for clarification.

- It would make it much easier for reviewers if the Figure number could be noted on the figure especially for the extended material, thank you.

Response (3.11): Yes, we have added the figure number to each figure in this revised version.

Referee #4:

The authors investigate skin cell behavior in vivo with focus on cell expansion to achieve appropriate body coverage. They have employed an innovative system of multicolor barcoding of cells combined with state-of-the-art quantitative imaging to study the dynamics of zebrafish skin development in vivo. The key new finding underlying the manuscript is that terminally-differentiated skin epithelial cell populations can increase in number through a process termed "asynthetic fission", i.e. cell division without DNA duplication. Such divisions are well known in the meiotic division cycle as well as in some polyploid cell types (in particular liver cells), however, asynthetic fission is not established in relation to skin cell behavior.

Response (4.1): We thank the referee for these comments and would like to provide our view on the cell division process we described in this study. Based on additional analyses and experiments, we respectfully suggest that "asynthetic fission" differs from typical mitosis or meiosis due to the following key features: (1) individual progeny cells become smaller and thinner upon each division; (2) DNA content is proportionally reduced upon each division; (3) the division process at the DNA level is error-prone, as chromosome segregation defects (e.g., "anaphase bridges", "lagging chromosomes", "micronuclei", and "tripolar segregation") are prevalent during SEC division. We have now included these important new results as Fig. 4m-q. in this revision.

Major points

1.) The authors suggest that in addition to the rapid supply of cells from stem- or progenitor cell populations, the differentiated cell population can also be "split" in these processes to rapidly support cell number demands. At this stage, it is unclear how relevant the described model will be across species and whether it reflects also the development of other non-keratinized stratified epithelium. Further, the authors do not analyze the molecular mechanisms underlying the fission process, which appears uncontrolled based on the current manuscript data. Obviously, uncontrolled fission is expected to be deleterious for cell fitness and function due to the unequal separation of the genome and cytoplasmic contents. The fission strategy is therefore counterintuitive, in fact, tissues of many organs (including epithelia) employ polyploidization as a strategy to cope with cell growth demands. In this regard, it is important for authors to ensure that SEC divisions take place in diploid cells rather than a subpopulation of polyploid cells.

Response (4.2): We fully agree with the referee that it is unclear how relevant our described model will be across different animal species and whether it might reflect the development of other non-keratinized stratified epithelia. We hope that the referee would agree with our opinion that these important questions do not reduce the value of our findings describing a variant form of cell division in a vertebrate tissue. Despite the current technological challenges in applying similar imaging technologies to other animal models, we are testing several alternative approaches and hope that we will be able to elaborate on whether this cell division mode also exists in other contexts in future studies.

Response (4.3): We apologize that the entire dataset regarding molecular mechanisms of the fission process were not shown in the main figures (presented as Extended Data Fig. 6 in the previous version). In this revised manuscript, we are excited to report that we have additional results to show the expression of Pizeo1 in the SEC population, and we have genetically tested the involvement of Piezo1 in SEC division. We have included the old and new results describing the involvement of Piezo1 in Fig. 6 and Extended Data Fig. 9, and revised the text on page 15, paragraph 1:

> "To determine whether Pizeo1 is indeed required for asynthetic fission, we applied a CRISPR/Cas9-based approach to disrupt Piezo1 expression in live zebrafish larvae. Injection of Piezo1 sgRNAs at the 1-cell stage effectively reduced Piezo 1 expression in vivo (decrease of 53%; Fig. 6j-l), leading to significant suppression of the SEC division event in 8 dpf palmskin (39% less; Fig. 6m-p). Of note, we detected no growth abnormality in the sgRNAs injected animals, in terms of body length or body surface area (Fig. 6q, r). We further determined that Piezo1 is present as cytoplasmic puncta in SECs regardless of growth-induced stretch (Extended Data Fig. 9g, h). Taken together, the data lead us to conclude that the activity of the stretch-activated ion channels, presumably including Piezo1, is key to stimulation of asynthetic fission."

Response (4.4): To determine whether the SEC population may contain polyploid cells, we performed additional flow-cytometry-based DNA content analyses on the SEC population at 8, 10, 14, 21 dpf and adult stages. We determined that the proportion of SECs in the S/G2/M phases was always below 10% across all examined time points (Fig. 4k). Since up to 60% of SEC cells may undergo division (Fig. 5k), we thus conclude that most SEC divisions take place in diploid cells. In addition, we have now performed DAPI staining to measure the nuclear DNA content in individual SECs. We determined that the DAPI signal per cell in 2- and 4-cell clones is proportionally reduced, consistent with our prior findings from the histone level quantification and the flow-cytometry-based DNA content analyses. We have included these new results in Fig.4a-c and 4k, and revised the text on page 9, paragraph 2 and page 10, paragraph 1.

> "If a somatic cell population could divide without DNA replication, one would expect the progeny cells to have a reduced genome size. To examine this idea, we first determined relative changes in genomic DNA content by DAPI staining in fixed tissues. Remarkably, we found that nuclear DAPI signals per cell were reduced proportionally in 2-cell and 4-cell SEC clone (34% and 60% less, respectively; Fig. 4a, b), indicative of a sequential reduction in DNA content. Of note, we determined that the nuclear DAPI signals are specific and not affected by nuclear BFP2 expression, as tissue fixation eliminated the BFP2 signal in full (Fig. 4c)."

> "Based on EdU assays, DAPI staining, histone titration, HU treatment, and flow-cytometry-based DNA content analyses, we termed this cell division mode 'asynthetic fission', as the process appears to require little or no DNA synthesis for producing more cells."

2) The authors are largely focused on the organismal in vivo aspects of asynthetic fission. Unfortunately, they do not include in depth analysis at the cellular levels to advance our

understanding of the highly interesting fission division process. Hence, the manuscript is descriptive and would benefit greatly from more careful analysis of SEC cells division. Particularly, the distribution of genomic material in the divided SEC cells should be addressed beyond the indirect assays applied in the manuscript. Flow cytometry analysis presented in figure 4g does not indicate clear haploid (1N) or hypo-haploid (0.5N) populations. This observation warrants further investigation to appreciate the distinction of the presented asynthetic fission form of cell division from mitotic (or meiotic) division. The presented systems should be amenable for deeper analysis by single cell sequencing. An alternative to single-cell sequencing could be karyotype analysis, preferably combined with multicolor FISH or similar.

Response (4.5): We thank the reviewer for this comment. To visualize the distribution of genomic content during SEC division, we created an additional transgenic line Tg(krt4:H2A-EGFP) to monitor DNA segregation activities in dividing SECs. Surprisingly, from the time-lapse images, we were able to capture several chromosome segregation defects, such as “anaphase bridges”, “lagging chromosomes”, “micronuclei”, and “tripolar segregation” in the dividing SECs. These live imaging results provide further evidence that SEC division does not proceed as typical mitosis or meiosis; instead, it is evidently highly error-prone. Thus, we speculate that the division is likely to be imprecise or non-symmetric, which could explain why the FACS profile does not include sharp peaks representing cells that have either a 1N or 0.5N DNA content. We have included these important new results as Fig. 4m-q, and Supplementary Video 8 and 9, updated the Methods section and the text on page 10, paragraph 2:

> “To monitor the nuclear division process in SECs in real time, we created a transgenic line, Tg(krt4:H2A-EGFP), to capture chromosome segregation dynamics during SEC division. Remarkably, we identified frequent chromosome segregation defects in the dividing SECs but not in the BECs, including “anaphase bridges”, “lagging chromosomes”, “micronuclei”, and “tripolar segregation” (Fig. 4m, n; Supplementary Video 8, 9). Time-lapse imaging of double transgenic animals further showed that the division process is markedly lengthened in SECs, as determined by the time spent from prophase to telophase (70.2 ± 18.22 min in BECs vs 108.3 ± 26 min in SECs; n = 97 captured events; Fig. 4m, o, p; Methods).”

Response (4.6): The question regarding how exactly do the genomic material is distributed between SEC cells is an important question. We have assembled a team of researchers to determine how we may perform single-cell DNA sequencing on SECs. Despite the need for customizing single-cell DNA sequencing probes for zebrafish cells (Mission Bio's platform), we also realized that a full set of bioinformatics algorithms and analysis pipelines will need to be developed if we would like to unambiguously sort out each of 25 chromosomes in 1-cell, 2-cell, and 4-cell clones. Furthermore, if the DNA segregation is completely random during the error-prone process (Response 4.5), we will need to overcome certain statistical challenges and background noise that is intrinsic to single-cell sequencing data. Thus, we respectfully request that the referee allow us to pursue this important direction in an in-depth future study.

Response (4.7): As suggested, we performed the Fluorescence in situ hybridization (FISH) assay on SECs targeting specific chromosomes. However, we determined that the assay has a high false negative rate in control samples. As FISH assays are generally used for qualitative assessments in population-based sample surveys (i.e. whether additional chromosomes might ever exist in a sample), the approach often fails short for quantitative assessments (i.e. how many cells may have a missing chromosome). We hope the referee to allow us to omit these inconclusive assay results.

3) Connected with point 2., it is also important for authors to characterize the division process in at least some detail since this process is the key discovery in the manuscript. Presumably, the dividing SEC cells go through stages of chromosome condensation, segregation, and cytokinesis. The authors should analyse fundamental characteristics of the mitotic division machinery such as centrosome, spindle, and kinetochore formation and behavior. Furthermore, basic questions along these lines have not been addressed. How does interference with chromosome segregation or cytokinesis impact the SEC population? Under these conditions respectively can mitotically trapped or binucleated SEC cells be identified?

3) Connected with point 2., it is also important for authors to characterize the division process in at least some detail since this process is the key discovery in the manuscript. Presumably, the dividing SEC cells go through stages of chromosome condensation, segregation, and cytokinesis. The authors should analyse fundamental characteristics of the mitotic division machinery such as centrosome, spindle, and kinetochore formation and behavior. Furthermore, basic questions along these lines have not been addressed. How does interference with chromosome segregation or cytokinesis impact the SEC population? Under these conditions respectively can mitotically trapped or binucleated SEC cells be identified?

Response (4.8): We thank the reviewer for this comment. As mentioned above in Response (4.5), we have now visualized the entire SEC division process using a newly created transgenic line Tg(krt4:H2A-EGFP), which allowed us to identify “anaphase bridges”, “lagging chromosomes”, “micronuclei”, and “tripolar segregation” in dividing SECs (Fig. 4m-p and Supplementary Video 8, 9). Also, as suggested, we have performed immunostaining on whole-mount fixed palmskin animals with antibodies against alpha-tubulin (mitotic spindles) and gamma-tubulin (centrosomes). Remarkably, we identified “tripolar spindles” in the dividing SECs at a high ratio (20%; n = 26/128). In sharp contrast, we detected no such spindle abnormalities in any of the dividing BECs (0%; n = 0/108), i.e., the skin stem cell population. We have included these important new results in Fig. 4q and updated the text on page 10, paragraph 2:

> “Notably, immunostaining of mitotic spindles and centrosomes further revealed many dividing SECs with “tripolar spindles”. We saw no such spindle abnormalities in any of the dividing BECs (0% in BECs vs. 20% in SECs; n = 0/108 and 26/128 cells, respectively; Fig. 4q).”

Response (4.9): To determine how interference with chromosome segregation or cytokinesis might impact the SEC population, we conducted a small-scale chemical screen to determine whether and how CDK and other cell cycle inhibitors may influence the process. In brief, we treated palmskin

animals with HU, RO3306, Paclitaxel, Roscovitine, and Nocodazole for 12 hours at 9.5 dpf. Intriguingly, we found that SEC division is only highly sensitive to the tubulin polymerization inhibitor, Nocodazole. The treatment caused a significant reduction in the divided pool and a marked increase in binucleated cells, just as the referee has predicted. We have now included these new results in Extended Data Fig. 6, updated the Methods section, and revised the text on page 10, paragraph 2:

> "To determine whether common cell cycle machineries may mediate asynthetic fission, we tested the effects of a panel of cell cycle inhibitors. Intriguingly, we determined that the division process is only highly sensitive to the treatment of the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6). Thus, we conclude that unlike mitosis or meiosis, asynthetic fission is error-prone, with a putative absence of well-orchestrated chromosome alignment during the division process."

4) Furthermore, the claim of DNA replication absence in SEC cells is not fully reflected in the data. It appears in ED 4d that no replication was observed in SEC cells, however, this is using short EdU pulse. Notably, the replication block experiments with more long-term HU in fact indicate a very substantial role of DNA replication as seen in ED 4j-k (authors acknowledge this in very modest fashion stating ", the impact of HU treatment on SEC division was relatively moderate."). Yet, this finding is not addressed, rather, authors focus very much on replication-independent fission divisions. Authors need to expand on this key point experimentally. This can be by evaluating DNA replication by other means than EdU, applying additional (non-HU) replication inhibitors, as well as applying additional time-series analysis.

Response (4.10): We thank the reviewer for the comment and would like to provide our view on this matter. We respectfully suggest that the EdU assay is perhaps the most direct, most sensitive assay for assessing DNA replication on a large scale. As an internal control reflecting the sensitivity of the assay, a significant amount of DNA replication was observed in the stem cell BEC population (i.e. 1.3% to 4.7% of a total of 37,516 BEC cells). Using the same assay, we did not detect even a single EdU positive cell in the SEC population from a survey of 17,809 cells in 50 animals. Thus, we acknowledge the possibility that a very low level of DNA replication may occur in SEC cells. We would like to emphasize that collective evidence from the EdU assay, direct DNA staining (the DAPI assay), quantification of histone levels, and the flow-cytometry-based DNA content assays all support the conclusion that DNA replication probably does not occur in SECs or it only occurs at a minimal level that is below the detection limits of our available methods. We have now revised the text on page 10, paragraph 1 to comment on this point:

> "Based on EdU assays, DAPI staining, histone titration, HU treatment, and flow-cytometry-based DNA content analyses, we termed this cell division mode 'asynthetic fission', as the process appears to require little or no DNA synthesis for producing more cells."

Response (4.11): As we determined that SEC division is tightly coupled with the animal growth rate (Fig. 5), the effect of HU (or other DNA replication inhibitors) on the SEC division is likely complicated

by its general influence on animal growth (i.e. no cell proliferation, no animal growth). For clarification, we have revised the text regarding the HU effect on SEC division on page 8, paragraph 2:

> "Despite its elimination of nearly all EdU-positive BECs (~97%; Extended Data Fig. 5e-g), the HU treatment failed to block SEC division to a similar extent."

Response (4.12): We thank the reviewer for the comment. We have conducted a time-series analysis with the EdU assay at 6, 8, 10, and 14 dpf. The results are consistent across all examined time points and shown as Extended Data Fig. 5c and 5d.

5) In figure 4 f-h, the authors show a substantial increase in "subG1" SEC cells, however in this experiment it is unclear from which population of cells these are arising from as there is no clear loss of the G1 population. Continuous supply of "G1" SEC cells may account for this. Potentially, similar analysis of HU treated samples, which the authors indicate prevents BEC cell division but not SEC cells, could reveal this population and strengthen the presented observations.

Response (4.13): We thank the reviewer for the suggestion. SEC division is tightly coupled with animal growth (Fig. 5) and the HU treatment globally affects DNA replication, so it also affects the animal growth rate. We suggest that the HU treatment is unlikely to enrich the divided pool. Of note, we would like to highlight that we have now performed a DAPI staining assay (Response 4.4) on whole-mount fixed palmskin animals to compare genomic DNA content per cell in 1-cell, 2-cell, or 4-cell clones. The results are consistent with the findings from our independent quantification of histone levels in live animals and the flow-cytometry-based DNA content analyses.

6) Recently it was shown that tamoxifen binds to the sodium channel and impairs its recovery from the inactivated state, resulting in fewer channels available for reopening by subsequent depolarizations (https://doi.org/10.1016/j.molcel.2020.12.048). Given the suggested role of ion channels in the regulation of superficial epithelial cells (SECs) division, could tamoxifen treatment used for barcoding induction affect the observed process?

Response (4.14): We thank the reviewer for providing this reference. We think that the tamoxifen treatment should not affect the SEC division process for two reasons. First, for barcoding SEC cells, we treated fish with tamoxifen only for a short time period (i.e. 2 hours) at 4 days post fertilization (dpf). As we determined that SEC division occurs most frequently from 8 to 14 dpf, the division is unlikely to be affected by the treatment at 4 dpf. Second, without tamoxifen treatment, we readily captured the division event in two independently created transgenic lines: Tg(krt4:NLS-EGFP) and Tg(krt4:H2A-EGFP) (Fig. 4i-4l and 4m-4q). Thus, we conclude that the tamoxifen treatment has little to no influence on SEC division.

Minor points.

1. The approach presented in 4f-h largely excludes dying cells from the analysis however the terminology used "subG1" is commonly used to indicate an apoptotic cell population by flow cytometry. The authors should consider using a term such as sub-2N or sub-diploid in the figures and text to avoid confusion to the readers.

Response (4.15): We thank the reviewer for this excellent suggestion. We fully agree that “sub-2N” is a much better term to use in this context. As suggested, we have now revised the figures (Fig. 4i, 4l, and Extended Data Fig. 8f) and the text (page 10, paragraph 1) to avoid confusion.

2. The presentation of FACS cell cycle profiles/DNA content is not optimal. In 4f, on the scatter plot authors gate (red square) for sub-2N (a much better term than sub-G1), but then authors show classical cell cycle histogram with 2N and 4N (and no sub-2N), with no explanation in figure legend.

Response (4.16): As suggested, we have now revised the figures (Fig. 4i and Extended Data Fig. 8f) and the figure legends as suggested.

3. In ED 4c, the EdU stain is seen in multiple samples and not always in the nucleus. What is the nature of the signal if not replication? At least in ED 4f, EdU signal was completely dependent on nucleotide supply as judged by the HU impact.

Response (4.17): We thank the reviewer for their careful reading. In ED 4c, we performed the EdU staining assay on specific transgenic reporter lines that label either the SEC nuclei or the BEC nuclei. So EdU signals that are not co-localized with the fluorescent-protein tagged SEC nuclei or the BEC nuclei are from “non-SEC” or “non-BEC” cells. For clarification, we have now added additional arrows to highlight those signals, as they indicate that the EdU assay is working properly. We also revised the text in the figure legend to avoid confusion.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed most of the comments of this reviewer and the manuscript has substantially improved. The evidence that divisions without replication occur in the suprabasal layer allowing surface expansion of single cells without the need to produce more plasma membrane is now quite compelling. However, I am somewhat puzzled about the new stretch-induced ion channel data. In the Piezo knockdown, despite reducing division numbers, there is no effect on body length, which would be predicted if stretch induced asynthetic fission would be essential to couple SEC expansion to body growth. Further along these lines, looking at the quantification for Fig. 5 f,g it seems that the relative amount of 1-cell clones and % divided cells in gadolidium treated animals, where divisions are blocked, is in fact almost similar to the vehicle treated condition on its right (which serves as a control for Yoda). This raises the question on the robustness of the division phenotype, its direct relation to body growth as well as its essential vs non-essential function. The fact that in some cases 85% of the cells have not divided (vehicle control condition to Yoda) might also explain the infrequency of EdU-labelled cells but the strong effect of HU on cell divisions (a concern in the previous version, where statistical analyses indeed confirms the substantial effect on division). Can the authors exclude that cell division rates are highly variable and division can occur both in a synthetic and non-synthetic manner and that these modes are flexible? Would be helpful if the authors could clarify these points prior to publication. As suggested by another reviewer in the previous round, a second means to block replication would be helpful. In addition, it might be useful to examine the effect of HU on total axis growth as it might be that the effect of HU on SEC divisions is indirect and results from an overall impact on growth. This could reduce tension in the SEC layer thereby limiting the need for asynthetic division.

Referee #2 (Remarks to the Author):

The authors have done a brilliant job of addressing the reviewers' many comments. The paper is much much better as a result. The new detailed analyses of divisions (spindles/cell shape/DNA content) are excellent. This is a fascinating study that reports important findings and I fully support it making it into print.

Referee #3 (Remarks to the Author):

In the revised version of the manuscript the authors did a good job in adding new important experiments, reshuffling figures and clarifying their points. Especially the imaging of spindles and the further characterization of the divisions improve the study. As does the additional data and new figure on the mechano-sensing hypothesis.
This study now is an elegant, thorough characterization of a new division mode first observed for developing zebrafish skin epithelia, but possibly more widespread. But even if not widespread, it is a

fascinating phenomenon that deserves recognition. As the authors mention themselves, many important questions remain open and need further investigation, but I do agree with them that this is beyond the scope of this study and they have now clarified this better in the discussion.

However, before publication two additional points need to be added/clarified:

1) I could not find neither in text, nor figure legends or material and methods how the efficiency of the drug conditions were assessed. This is particularly important for the drugs that did not show an effect. For example, while it is not surprising that nocodazole has an effect, as divisions seem to need spindles, it is slightly puzzling that Taxol did not give a similar phenotype. Positive controls for all drug conditions must be shown at similar stage and environment as the experiments performed. This is particularly important as in zebrafish drugs can have very variable effects and sometimes readout, especially when no phenotype is observed, can be hard.

2) Text additions:
The prolonged telophase is an interesting finding. It does contradict however the assumption that these cells do not react to any DNA control checkpoints. Can the authors briefly speculate about this conundrum and what it might mean for asynthetic fission?

Referee #4 (Remarks to the Author):

Chan and colleagues have generally addressed the review report points in a manner that improves the manuscript, which includes addition of new data to further expand on the asynthetic fission process. However, a few points remain as outlined below in this report. Furthermore, the authors have clarified and justified important issues that were identified such as precision documentation of distribution of genetic content to the progeny cells resulting from the fission process. Finally, due to the obvious fitness issues of progeny, asynthetic fission is expected to be a very uncommon approach to solve specific challenges that may be most prominent in tissues where cell shedding occurs, such as developing skin.

Comments:
-Author Response (4.1): We thank the referee for these comments and would like to provide our view on the cell division process we described in this study. Based on additional analyses and experiments, we respectfully suggest that “asynthetic fission” differs from typical mitosis or meiosis due to the following key features: (1) individual progeny cells become smaller and thinner upon each division; (2) DNA content is proportionally reduced upon each division; (3) the division process at the DNA level is error-prone, as chromosome segregation defects (e.g., “anaphase bridges”, “lagging chromosomes”, “micronuclei”, and “tripolar segregation”) are prevalent during SEC division. We have now included these important new results as Fig. 4m-q. in this revision.
***Reviewer comment to 4.1.: The more in-depth characterization of the division process is appreciated. However, the representative images of segregation defects lacks sufficient data support (Fig. 4m-q.). Thus, the new data should be accompanied with appropriate quantification and statistical analysis (beyond spindle abnormalities).

-Author Response (4.9): To determine how interference with chromosome segregation or cytokinesis might impact the SEC population, we conducted a small-scale chemical screen to determine whether and how CDK and other cell cycle inhibitors may influence the process. In brief, we treated palmskin animals with HU, RO3306, Paclitaxel, Roscovitine, and Nocodazole for 12 hours at 9.5 dpf. Intriguingly, we found that SEC division is only highly sensitive to the tubulin polymerization inhibitor, Nocodazole. The treatment caused a significant reduction in the divided pool and a marked increase in binucleated cells, just as the referee has predicted. We have now included these new results in Extended Data Fig. 6, updated the Methods section, and revised the text on page 10, paragraph 2:

“To determine whether common cell cycle machineries may mediate asynthetic fission, we tested the effects of a panel of cell cycle inhibitors. Intriguingly, we determined that the division process is only highly sensitive to the treatment of the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6). Thus, we conclude that unlike mitosis or meiosis, asynthetic fission is error-prone, with a putative absence of well-orchestrated chromosome alignment during the division process.”

***Reviewer comment to 4.9.: The non-significant effect of CDKs inhibitors on asynthetic fission is highly surprising as the positive-feedback increase in CDK activity is canonically required for cells to initiate cell division. It is textbook knowledge that increasing CDK activity mediates centrosome separation and maturation, nuclear envelope breakdown, and chromosome condensation. Notably, the authors document that these processes take place, however, CDK activity is suggested to be dispensable. How do the authors explain this surprising observation? It is plausible that CDK inactivation was not achieved by the authors? The authors should compare the phospho-markers of CDK activity between BEC and SEC dividing cells, and eventually also use them as a control for efficient CDK inhibition.

Author Rebuttals to First Revision:

Point-by-point responses

Referee #1:

Referee #1 (Remarks to the Author):

The authors have addressed most of the comments of this reviewer and the manuscript has substantially improved. The evidence that divisions without replication occur in the suprabasal layer allowing surface expansion of single cells without the need to produce more plasma membrane is now quite compelling. However, I am somewhat puzzled about the new stretch-induced ion channel data. In the Piezo knockdown, despite reducing division numbers, there is no effect on body length, which would be predicted if stretch induced asynthetic fission would be essential to couple SEC expansion to body growth. Further along these lines, looking at the quantification for Fig. 5 f,g it seems that the relative amount of 1-cell clones and % divided cells in gadolidium treated animals, where divisions are blocked, is in fact almost similar to the vehicle treated condition on its right (which serves as a control for Yoda). This raises the question on the robustness of the division phenotype, its direct relation to body growth as well as its essential vs non-essential function. The fact that in some cases 85% of the cells have not divided (vehicle control condition to Yoda) might also explain the infrequency of EdU-labelled cells but the strong effect of HU on cell divisions (a concern in the previous version, where statistical analyses indeed confirms the substantial effect on division). Can the authors exclude that cell division rates are highly variable and division can occur both in a synthetic and non-synthetic manner and that these modes are flexible? Would be helpful if the authors could clarify these points prior to publication.

As suggested by another reviewer in the previous round, a second means to block replication would be helpful. In addition, it might be useful to examine the effect of HU on total axis growth as it might be that the effect of HU on SEC divisions is indirect and results from an overall impact on growth. This could reduce tension in the SEC layer thereby limiting the need for asynthetic division.

Response (1.1): We thank the reviewer for the comment and would like to explain our view on the Piezo1 knockdown results. Because the role of Pizeol1 is to mediate the effects of tension on SEC division, we do not expect the Pizeol1 knockdown alone (which will decouple tension and division) to have a direct influence on animal growth and/or the tension level. To clarify this point, we have revised the text on page 15, paragraph 2:

> "Taken together, the data lead us to conclude that the activity of stretch-activated ion channels, presumably including Piezo1, is key to coupling the skin tension level with asynthetic fission."

Response (1.2): Because chemical treatments may affect both asynthetic fission and animal growth, we examined the effects of Gd and Yoda1 on asynthetic fission at early stages when animals have moderate growth (i.e., 8 to 10 dpf). Thus, the divided pool ratios in the control groups may seem variable (Fig. 6f and 6g). We would like to point out that as many as 50% of the SECs can opt for this type of division at 14 dpf (Fig. 4l), and yet, we failed to detect even a single EdU-positive cell in the SEC population across all examined time-points (i.e., 6, 8, 10, and 14 dpf; a survey of 17,809

cells in 50 animals; Extended Data Fig. 5d). Thus, we conclude that SEC division does not occur both with and without DNA synthesis. As suggested by the reviewer, we have now highlighted the EdU results on page 8, paragraph 2, and revised the text on page 10, paragraph 1 to clarify this point:

> "After a 1-hr incubation with EdU, we readily detected significant percentages of EdU-positive cells among BECs at 6, 8, 10, and 14 dpf (1.3% to 4.7%; n = 37,516 cells from 50 animals, collectively; Extended Data Fig. 5c, d), consistent with their role as stem cells in developing skin. In sharp contrast, we failed to detect any EdU-positive SECs from a survey of 17,809 cells in 50 animals over the same growth period (Extended Data Fig. 5c, d), indicating their terminally differentiated status."

> "Taken together, these observations led us to conclude that SEC division is a distinct and dynamic event that occurs most frequently during a narrow developmental period. Based on EdU assays, DAPI staining, histone titration, HU treatment, and flow-cytometry-based DNA content analyses, we termed this cell division mode 'asynthetic fission', as the process appears to require little or no DNA synthesis for producing more cells."

Response (1.3): As suggested, we examined the effects of another DNA replication inhibitor, AraC, on asynthetic fission. We first determined that a brief, 12-hr treatment of AraC is effective in blocking overall DNA replication (75% reduction; Extended Data Fig. 6b). However, the treatment does not affect animal growth or SEC division. In addition, we examined the effect of HU on animal growth as advised. We determined that a 12-hr treatment of HU is rather effective at blocking DNA replication (99% reduction; Extended Data Fig. 6a), yet the treatment has no apparent influence on animal growth and SEC division. We have included these new results as Extended Data Fig. 6e and 6j-6n, and updated the text on page 11, paragraph 1:

> "Intriguingly, despite each of the treatments markedly reducing cell proliferation (Extended Data Fig. 6a-e), we found that the SEC division process is most sensitive to treatment with the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6f-n). Of note, we kept the treatments brief (only 12 hr) to avoid an overt impact on animal growth, so these findings do not exclude a role for cell cycle checkpoints or CDKs during SEC division."

Referee #2 (Remarks to the Author):

The authors have done a brilliant job of addressing the reviewers' many comments. The paper is much much better as a result. The new detailed analyses of divisions (spindles/cell shape/DNA content) are excellent. This is a fascinating study that reports important findings and I fully support it making it into print.

Response (2.1): We thank the reviewers for their time and comments, which helped to greatly improve our work.

Referee #3 (Remarks to the Author):

In the revised version of the manuscript the authors did a good job in adding new important experiments, reshuffling figures and clarifying their points. Especially the imaging of spindles and the further characterization of the divisions improve the study. As does the additional data and new figure on the mechano-sensing hypothesis.

This study now is an elegant, thorough characterization of a new division mode first observed for developing zebrafish skin epithelia, but possibly more widespread. But even if not widespread, it is a fascinating phenomenon that deserves recognition. As the authors mention themselves, many important questions remain open and need further investigation, but I do agree with them that this is beyond the scope of this study and they have now clarified this better in the discussion.

However, before publication two additional points need to be added/clarified:

1) I could not find neither in text, nor figure legends or material and methods how the efficiency of the drug conditions were assessed. This is particularly important for the drugs that did not show an effect. For example, while it is not surprising that nocodazole has an effect, as divisions seem to need spindles, it is slightly puzzling that Taxol did not give a similar phenotype. Positive controls for all drug conditions must be shown at similar stage and environment as the experiments performed. This is particularly important as in zebrafish drugs can have very variable effects and sometimes readout, especially when no phenotype is observed, can be hard.

Response (3.1): We thank the reviewer for the encouraging comments on the study, and we apologize for not clearly describing the inhibitor treatment conditions. We have revised the text in the Methods to list the references we followed for each of the inhibitor treatment conditions (page 22, paragraph 2). In addition, as suggested by the reviewer, we examined and verified the treatment effects using the EdU assay. We determined that HU, AraC, Roscovitine and Nocodazole all effectively reduce the number of EdU+ cells when treated at the same developmental stages and concentrations as used in the experiments (12-hr treatment at 9.5 dpf; n = 3 independent biological replicates). Of note, we have omitted inhibitors that failed to affect cell proliferation in 12 hours in order to avoid over-interpretation of the results. We have now included these important positive controls as Extended Data Fig. 6a-e.

2) Text additions:

The prolonged telophase is an interesting finding. It does contradict however the assumption that these cells do not react to any DNA control checkpoints. Can the authors briefly speculate about this conundrum and what it might mean for asynthetic fission?

Response (3.2): The drug treatments can affect both animal growth and SEC division, and these two processes are coupled. Therefore, we treated the animals with cell cycle inhibitors only for a short time (i.e., 12 hr). Based on the drug treatments alone, we cannot speculate that asynthetic fission does not react to DNA control checkpoints. We can only conclude that the division is most sensitive to the tubulin polymerization inhibitor. In fact, as pointed out by the reviewer, the prolonged

telophase suggests that these cells should still react to DNA control checkpoints. We thank the reviewer for this question, and we have revised the text to point out the limitations of this exploratory experiment on page 11, paragraph 1:

> "Intriguingly, despite each of the treatments markedly reducing cell proliferation (Extended Data Fig. 6a-e), we found that the SEC division process is most sensitive to treatment with the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6f-n). Of note, we kept the treatments brief (only 12 hr) to avoid an overt impact on animal growth, so these findings do not exclude a role for cell cycle checkpoints or CDKs during SEC division."

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Chan and colleagues have generally addressed the review report points in a manner that improves the manuscript, which includes addition of new data to further expand on the asynthetic fission process. However, a few points remain as outlined below in this report. Furthermore, the authors have clarified and justified important issues that were identified such as precision documentation of distribution of genetic content to the progeny cells resulting from the fission process. Finally, due to the obvious fitness issues of progeny, asynthetic fission is expected to be a very uncommon approach to solve specific challenges that may be most prominent in tissues where cell shedding occurs, such as developing skin.

Comments:

-Author Response (4.1): We thank the referee for these comments and would like to provide our view on the cell division process we described in this study. Based on additional analyses and experiments, we respectfully suggest that "asynthetic fission" differs from typical mitosis or meiosis due to the following key features: (1) individual progeny cells become smaller and thinner upon each division; (2) DNA content is proportionally reduced upon each division; (3) the division process at the DNA level is error-prone, as chromosome segregation defects (e.g., "anaphase bridges", "lagging chromosomes", "micronuclei", and "tripolar segregation") are prevalent during SEC division. We have now included these important new results as Fig. 4m-q. in this revision.

****Reviewer comment to 4.1.: The more in-depth characterization of the division process is appreciated. However, the representative images of segregation defects lacks sufficient data support (Fig. 4m-q.). Thus, the new data should be accompanied with appropriate quantification and statistical analysis (beyond spindle abnormalities).*

Response (4.1): As suggested, we have now included additional statistical analysis of the prevalence of chromosome segregation defects during SEC division (Fig. 4r and 4s).

-Author Response (4.9): To determine how interference with chromosome segregation or cytokinesis might impact the SEC population, we conducted a small-scale chemical screen to determine whether

and how CDK and other cell cycle inhibitors may influence the process. In brief, we treated palmskin animals with HU, RO3306, Paclitaxel, Roscovitine, and Nocodazole for 12 hours at 9.5 dpf. Intriguingly, we found that SEC division is only highly sensitive to the tubulin polymerization inhibitor, Nocodazole. The treatment caused a significant reduction in the divided pool and a marked increase in binucleated cells, just as the referee has predicted. We have now included these new results in Extended Data Fig. 6, updated the Methods section, and revised the text on page 10, paragraph 2:

"To determine whether common cell cycle machineries may mediate asynthetic fission, we tested the effects of a panel of cell cycle inhibitors. Intriguingly, we determined that the division process is only highly sensitive to the treatment of the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6). Thus, we conclude that unlike mitosis or meiosis, asynthetic fission is error-prone, with a putative absence of well-orchestrated chromosome alignment during the division process."

****Reviewer comment to 4.9.: The non-significant effect of CDKs inhibitors on asynthetic fission is highly surprising as the positive-feedback increase in CDK activity is canonically required for cells to initiate cell division. It is textbook knowledge that increasing CDK activity mediates centrosome separation and maturation, nuclear envelope breakdown, and chromosome condensation. Notably, the authors document that these processes take place, however, CDK activity is suggested to be dispensable. How do the authors explain this surprising observation? It is plausible that CDK inactivation was not achieved by the authors? The authors should compare the phospho-markers of CDK activity between BEC and SEC dividing cells, and eventually also use them as a control for efficient CDK inhibition.*

Response (4.2): We thank the reviewer for the comment and would like to provide our interpretation on this issue. Based on the drug treatments alone, we do not propose or conclude that canonical CDK activity is dispensable for asynthetic fission. In fact, we found that the division process is lengthened in SECs in contrast to BECs (Fig. 4o, 4p). This finding suggests that CDK activity or cell cycle checkpoints should still have a role in SEC division. Of note, we have now determined that each of the listed inhibitors is effective (new Extended Data Fig. 6a-e). In addition, we have revised the text on page 11, paragraph 1 to point out the limitations of this drug treatment experiment:

> "Intriguingly, despite each of the treatments markedly reducing cell proliferation (Extended Data Fig. 6a-e), we found that the SEC division process is most sensitive to treatment with the tubulin polymerization inhibitor, Nocodazole, which caused a significant reduction in the divided pool and a marked increase in binucleated cells (Extended Data Fig. 6f-n). Of note, we kept the treatments brief (only 12 hr) to avoid an overt impact on animal growth, so these findings do not exclude a role for cell cycle checkpoints or CDKs during SEC division."

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed most of my remaining points, but I still fail to understand the lack of effect of uncoupling growth-driven tension sensing and cell division. As I understand, that authors model is that organismal growth generates tension on the epithelium, this tension then acts through Piezo to trigger divisions to couple skin growth to organismal growth. This is summarized in the abstract by the statement “Taken together, our findings introduce a distinct type of cell division event in the development of a vertebrate species that enables large-scale skin expansion on an as-needed basis”.
By disrupting tension sensing through inhibition of Piezo they see less cell division, which according to the above logic should prevent skin expansion to match organismal growth. Thus it is difficult for me to understand how absence tension induced, asynthetic mitosis-driven skin expansion to match organismal growth would have no effect on the organism unless this pathway is not essential and the skin is capable of expansion through another mechanism. The “as needed-basis” of expansion still exists as the organism grows normally and thus the skin presumably normally expands in the Piezo-inhibited conditions where asynthetic mitosis is compromised. The authors response to this critical point remains vague and the conclusions on the relevance of this mechanism thus appear overstated.

Referee #3 (Remarks to the Author):

The authors addressed all my remaining comments and I congratulate them to their study that should now be ready for publication.

Referee #4 (Remarks to the Author):

The authors have addressed the points that were remaining in a reasonable manner. The functional CDK inhibitory data are hard to interpret - the minor impact on the divided cell pool is unexpected, possibly it's a timing issue. At the level of the individual data points, it seems that sometimes CDK inhibition actually might have some effect, hence, the big spread of values...(Ext.Fig.6K). However, it is now a minor issue given that authors toned down the interpretation of this experiment in the text.

Author Rebuttals to Second Revision:

Point-by-point responses

Referee #1 (Remarks to the Author):

The authors have addressed most of my remaining points, but I still fail to understand the lack of effect of uncoupling growth-driven tension sensing and cell division. As I understand, that authors model is that organismal growth generates tension on the epithelium, this tension then acts through Piezo to trigger divisions to couple skin growth to organismal growth. This is summarized in the abstract by the statement "Taken together, our findings introduce a distinct type of cell division event in the development of a vertebrate species that enables large-scale skin expansion on an as-needed basis".

By disrupting tension sensing through inhibition of Piezo they see less cell division, which according to the above logic should prevent skin expansion to match organismal growth. Thus it is difficult for me to understand how absence tension induced, asynthetic mitosis-driven skin expansion to match organismal growth would have no effect on the organism unless this pathway is not essential and the skin is capable of expansion through another mechanism. The "as needed-basis" of expansion still exists as the organism grows normally and thus the skin presumably normally expands in the Piezo-inhibited conditions where asynthetic mitosis is compromised. The authors response to this critical point remains vague and the conclusions on the relevance of this mechanism thus appear overstated.

Response (1): We thank the reviewer for the comment and would like to point out that zebrafish skin at this particular developmental stage is a stratified, bi-layered structure. As the asynthetic fission-directed mechanism only accounts for skin expansion in the top layer (i.e., the SEC population), the basal stem cell layer (i.e., the BEC population) can still undergo classical mitosis to support animal growth and/or skin expansion when the mechanism is compromised (i.e., under Piezo1 inhibition). Thus, we believe our statement in the Abstract is valid for skin cells in the top layer. To better explain the anatomical structure of fish skin and to point out the limitations of our findings in other aspects, we have now revised the Discussion on page 12, paragraph 3, and page 13, paragraph 1:

> *"To maintain a stratified structure, the skin progenitor cells residing in the basal layer (BECs) can undergo classical mitosis to keep up with growth demands[31]. Yet, this option is unavailable to terminally differentiated epithelial cells, such as SECs. Through asynthetic fission, skin cells devoid of mitotic potential may retain a means of producing more apical surface area as the larval skin gradually transitions from a bi-layered structure to a multi-layered epidermis[13]."*
>
> *"It also remains to be determined whether defects in asynthetic fission would compromise skin barrier function and whether the process is evolutionarily conserved in mammals[15,38,39]."*

Referee #3 (Remarks to the Author):

The authors addressed all my remaining comments and I congratulate them to their study that should now be ready for publication.

Referee #4 (Remarks to the Author):

The authors have addressed the points that were remaining in a reasonable manner. The functional CDK inhibitory data are hard to interpret - the minor impact on the divided cell pool is unexpected, possibly it's a timing issue. At the level of the individual data points, it seems that sometimes CDK inhibition actually might have some effect, hence, the big spread of

values...(Ext.Fig.6K). However, it is now a minor issue given that authors toned down the interpretation of this experiment in the text.

Response (2): We thank the reviewers for their time and comments, which helped to greatly improve our work.