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Bimodal behaviour of interfollicular epidermal progenitors regulated by hair follicle position and cycling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 March 2016

Thank you for the submission of your manuscript (EMBOJ-2016-93806) to The EMBO Journal and for sending us the preliminary point-by-point response to the referees' reports (which I enclose below). My apologies for the extended duration of the review process in this case. As outlined earlier, two referees have seen your manuscript and they both highlight the potential interest and novelty of your study. However, at the same time the reviewers express reservations related to incomplete experimental documentation that makes the study inconclusive at the current stage. Moreover, both referees point to missing controls that would need to be included in the revised manuscript. In summary, the referees do not find the claims made sufficiently supported by the data at this stage and they therefore ask you to strengthen the study by both addressing currently unresolved issues, and carefully revising the technical documentation.

In addition to the original reports, referee #2 has also seen your point-by-point response outlining experiments to be included in a revised manuscript. While the referee finds that your response would go a long way to address the referee concerns this person emphasizes the need for you to focus on essential points in the study as outlined below. Please see below for details.

From my side, I judge the referee comments to be generally reasonable and we would therefore like to invite you to submit a revised manuscript addressing the referees' comments. Along these lines, I would like to ask you to please

• provide additional experimentation on open points
• provide additional control data on the methods

• revise the technical documentation of the experimental work
• re-write the manuscript to avoid over interpretation of the results

Since a large number of key experiments potentially impacting on the conclusiveness of the study have been suggested, and given the importance of the question addressed, it would be essential for you to provide a definitive and accurately described dataset in the revised version. We appreciate that the required revision could be beyond the scope of the current project and we would therefore understand if you chose to instead consider publication elsewhere.

Please contact me if you have any questions, need further input on the referee comments or if you consider engaging in a compelling revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this study the authors used a mouse model ("skinbow"), which allowed multicolor lineage tracing to investigate the clonal expansion of cells of the interfollicular epidermis (IFE). In particular, the authors aimed to examine the contribution of hair follicles and the hair renewal cycle to the turn over of the IFE specifically in back skin, which was not assessed in previous studies. The authors propose two modes of clonal expansion: 1) clones attached to hair follicles showed rapid increase in size during the growth phase of hair cycle; 2) clones distant from hair follicles were slow-cycling, but could be mobilized by proliferative stimuli. The "skinbow" mouse model could provide an interesting tool to study the clonal expansion of cells in the IFE of back skin. However, several claims are not supported by any data and the experimental design used in this study was often not suitable to convincingly prove the conclusions made by the authors.

There are major concerns and it is essential to address important issues to support the conclusions:

1) The authors used a tamoxifen-inducible mouse line and depending on the questions either a high or a low dose of tamoxifen was used. However, the authors do not provide all details and data necessary to support their claims and to demonstrate convincingly that the mouse model used in this study is suitable to address the proposed questions. The authors should provide further methodical details and present control experiments to exclude spontaneous recombination in the absence of tamoxifen, as claimed in the manuscript.

2) The manuscript is lacking elementary information regarding labeling efficiency and specificity of the approach. Along the same lines, the first time points of analysis post recombination (3 weeks, 5 weeks) are too late since cell clones might already be lost from epidermis by that time providing a modified and wrong set of data subsequently altering outcome of the modeling.

3) The authors injected mice either at P21 or p42 and aimed to label cells in growing mice after hair follicle formation was complete (P21) and in adult mice after the first hair cycle (P42). To convincingly show increased and rapid clonal expansion during the growth phase of hair follicles (anagen) it would be more important to label cells additionally in anagen phase (early anagen) and compare the clonal expansion during telogen and anagen. In most experiments, the authors harvested and analyzed the tissue 3 to 24 weeks after tamoxifen induction at P21 and P42. In the current setting, it is not clear how the authors distinguish between anagen and telogen hair follicles. The Wnt reporter is mentioned but no data are provided. The authors should choose more defined time points after tamoxifen induction, including time points allowing to systematically analyze initial labeling and clonal expansion.

4) The authors used FACS and confocal microscopy to study color distribution, number or size of clones. Using FACS alone, as done in Appendix Figure S2, seems to be an inaccurate way for

quantification of the different clones per color, since there is no clear separation of the different populations, representing the different colors. The authors should provide an additional quantification using confocal microscopy.

5) In the manuscript it is claimed, that small clones had cells in the basal layer of the epidermis in more than 80% of cases, with less than 20% being disconnected from the basal layer as expected in the case of clones undergoing terminal differentiation entirely and disappearing. The authors should support this conclusion by providing IF-co-staining for proliferation and differentiation marker.

6) Unfortunately, it is not clear how symmetric cell division versus asymmetric cell division was examined. The authors should provide details for this analysis and in addition use specific markers to examine spindle pool orientation during clonal expansion of labeled cells.

7) The label retention experiments are not convincing. The authors should systematically analyze efficiency of initial labeling and provide quantification data for proliferation and quiescence of keratinocytes. It is not clear which time points post BrdU pulse were examined (Figure 5).

8) To address the question if the detected slow-cycling cells respond to proliferative signals, the authors used TPA to stimulate proliferation and demonstrated a significant reduction in the proportion of small clones and a significant increase in large clones in TPA treated back skin. It would be interesting to demonstrate if the stimulation of proliferation induces more symmetric or asymmetric divisions. Furthermore, providing additional staining of a proliferation marker would be required to convincingly show that normally slow-cycling cells respond to TPA by increased proliferation and clonal expansion.

9) The authors propose an exchange between the two compartments of clones attached to hair follicle and clones not attached to hair follicles within three weeks. However the data provided here do not support this hypothesis and are over-interpreted. Would it not be expected anyway that larger clones eventually attach to or grow close to hair follicles given the space restriction between hair follicles?

In general, authors should carefully check that for each experiment the following information is provided: total dose/d; concentration of tamoxifen per ml; injection volume per kg mouse; number of injections and injection method (intra dermal or intraperitoneal). Furthermore, the authors should include the corresponding n-number of mice analyzed in figure legends. Finally, there seems to be a mistake in the graph of Figure 2 c (iii) and Figure 5 c: The bar representing large colony size (261 - over) is depicted differently within the graph itself and the figure legend.
In conclusion, the authors provide an interesting tool to study the clonal expansion of cells in the IFE of back skin. However, not all data are convincing and more data are required to support the author's conclusion. This study supports previous results published by other groups.

Referee #2:

1) In the submitted manuscript, the authors assess cellular behavior in mouse back skin based on quantitative fate mapping. They perform their analysis from 2 time points (p21 and p42) and reach the conclusion that interfollicular epidermis is maintained by cells with 2 different behaviours depending on their location. Cells that are associated (attached to HF) to hair follicles appear to be more proliferative than cells distant from hair follicles (not attached to HF). By computer simulation using a stochastic lattice model, the authors predict that anagen follicles influence cells in their immediate neighborhood (10-30 basal cells) to proliferative faster (0.1-0.3/week compared to 3/week).

Understanding the mechanisms that control tissue homeostasis is important and the submitted manuscript goes a long way in describing this. There is however a number of problems with the manuscript that needs to be addressed.

The title states that 'Homeostasis of the interfollicular epidermis is regulated by hair follicle cycling'. Firstly, homeostasis describes a tissue in equilibrium where cell loss equals cell gain, yet most of the analysis and conclusions are derived from analysis of data, where animals are labeled before they

reach adult size. There are significant differences between the 2 data sets (p21 vs p42) with much more prominent differences for the p21 dataset when assessing the differences between interfollicular epidermis that is either attached and non-attached hair follicles. Could expansion of the epidermis during mouse growth be driven by the areas closest to hair follicles?

2) Secondly, the authors state that the epidermis during anagen phase is proliferating more rapidly than during telogen. They show some images in figure 4 to support this notion. It is however unclear what the images represent.
A) Are these pictures obtained from adult animals?
B) Why is the epidermis in early anagen multilayered as if the skin has been treated with TPA or another compound?
C) Although the quantification appears solid the images provided to further substantiate the data (4A) are not representative of the quantification and there are no obvious Ki67 positive cells in the EMA, MA, C and T samples. The fractions should only be reduced.

3) In addition, it is hard to appreciate from Fig4C that cells closest to the hair follicle are proliferating more than those further away as suggested by the clonal fate data and the LRC analysis.

4) Moreover, if this is true the average clone size following the first anagen (w5 following p21 labeling) should be significantly greater in clones attached to HF compared to the non-attached ones (Figure 2a).

The authors spend a lot of effort discussing the importance of anagen on clonal growth, but they fail to provide any explanation why the average size of HF attached clones increase 4 fold between weeks 5 and 12 following labeling. Moreover, the effect of time in the P42 data set appears less prominent. How does the p42 data look if plotted like the p21 data set in Fig 2a?

5) The authors wish to show that the epidermis contains 2 distinct populations of cells characterized by different proliferation phenotypes. To address this they:
Firstly, perform a BrdU labeling experiment, where they inject animals repeatedly with BrdU at 21 days and chase for 5 weeks before analysis. There are multiple problems with these experiments since a cell will not take up BrdU unless it divides and they are therefore not likely to target very many of these cells. Moreover, BrdU is also killing fast cycling cells, which could affect tissue homeostasis. What does the tissue look like following 3 days of labeling and how many cells are actually labeled with BrdU? If cells far from the HF indeed are less proliferative a whole mount image as shown in figure 4c should illustrate this point nicely. To make solid conclusions of these observations, a thorough quantification should be completed. Moreover, the discussion of the data related to Merkel cells and touch domes is difficult to follow since there is no turnover in this compartment following development and BrdU should not label them.

6) Secondly, the authors perform TPA treatment and show that the fraction of small clones is reduced and state that this is evidence for their ability to contribute to homeostasis following injury. An alternative explanation is that a number of the small clones are lost during the TPA treatment leading to an apparent increase in the larger clones. This would fit with the observation that cells are streaming from the hair follicle into the interfollicular epidermis. Without loss of cells (clones) in the interfollicular epidermis, it is difficult to appreciate how the epidermis can accommodate the hair follicle influx. The experiment needs to be described in greater detail. Which mice are used? How old are they? Dose of TPA? Etc

7) The authors need to carefully consider their subtitles, as a number of them are misleading.
1. Multicolour lineage tracing for analysis of clonal population dynamics.
Are clones truly clonal? The authors also state that colour frequencies do not vary significantly over time. How has this been evaluated? Cerulean is increased >50%.

8) 2. Clone size evolution over time distinguishes two proliferation profiles.
The authors cannot state this based on their clonal fate data, where they observe some small clones and some bigger ones. The data is however very similar to that previously described by Jones and coworkers, and the authors go on to state that the clonal distribution is similar to that previously observed (Clayton et al., 2007, Nature), where the cells analyzed in tail epidermis could be modeled

based on one particular cell behavior. How can the authors then conclude that small clone population arises from cells that are slow cycling?
9) In the same section the authors state based on end point analysis that the some cell divide asymmetrically, whereas others divide symmetrically upon analysis of 2-4 cells clones. The authors cannot conclude that these are symmetric or asymmetric cell divisions giving rise to the outcome, as these end-point experiments relate to the final fate and not the outcome of the cell divisions. All the experimental data related to these experiments are missing (number of clones analyzed, distribution within the clones 2, 3 vs 4 cells, and the experimental details related to the timing)

10) Strategies to overcome clonal overlap
This is an odd title and is this really what the authors show? Firstly, it is unclear how a clone size > 7 cells at d3 is the only measure for merging 2 clones and qualifies clonal versus non-clonal. Some how the authors need to qualify their statement. Secondly, it is unclear how the elimination of some larger clones will qualify the assumption that two populations of cells exist. The authors have essentially just removed a number of events with preference for clones of size greater than an arbitrary value. Based on the committed progenitor model, analysis of tissues in homeostasis (not p21) one would expect a continuum of clonal sizes as the authors also observe. I am therefore unsure whether the authors can use this data as evidence for a two-population model.

11) They go on to look at rare populations (white, pink and cerulean) in the same experimental data set. Whereas White and pink seems to behave similarly, it is unclear whether cerulean label is representative (EV2). It is enriched more than 1.5 fold after 12 weeks in the labeling experiments (S2d) and clonal distribution is significantly different in the data provided for the single colors. In order to prove or disprove the single progenitor model the authors need to address whether their data display the same scaling behavior as described (Clayton et al., 2007; Mascre et al., 2012; Doupe et al., 2010)
Lastly, they state that they perform an experiment with low dose tamoxifen, but the authors choose not to share the data and the evidence that their analysis is indeed performed at clonal densities.

12) The authors go on to describe clones as attached to anagen and telogen areas. The data in Figure 3 is however very difficult to interpret, as the entire back skin should be in telogen by day 56 (5 weeks) followed by sporadic anagen phase in an asynchronous manner induced randomly.

13) The data for related to telogen versus anagen clones makes consequently little sense as this is just reflecting the current state of the epithelium and not necessarily how it has looked prior to the analysis. Moreover, the history related to whether one particular hair follicle has undergone single or multiple rounds of anagen is lost.

14) The authors describe growth of the organ by looking at distances between hair follicles (S4). How does this influence the shape of clones? Non-HF attached epidermis also expands more than HF attached? What about the size of the animal? Male versus female? How is this reflected in the lattice model? According to the measurements the mouse never reach homeostasis at least based on the structure of the unit measured. From which part of the mouse has these measurements been obtained?

The data related to the data simulation in EV5 is very difficult to comprehend as none of the simulations taking into account hair-follicle attachment (21 and 49) compared to no HF seems to be quantitatively better than the others. Some quantifiable measure for how one model is better than the other is required. Also, the similarity between experimental and modeled probabilities in EV5b is not that convincing. What does the model actually show? Something in between or that none of the proposed suggestions are correct.

Minor comments:
Page 3: Jones et al., 1995 never analyzed cells isolated from tissues but based their entire analysis on cultured keratinocytes

Figure 2A: The line connecting the values need to be removed and the data could potentially benefit from introduction of a log2 scale.

It will be helpful if the authors describe in detail what they measure. What does a clone of > 30 cells

represent? Is this basal and suprabasal cells

Additional comments referee #2 on authors' preliminary point-by-point response:

The following aspects need special attention:
1) Is there a hierarchy in the epidermis? Does cell around hair follicles really give rise to the cells further away. Can the authors truly conclude this? And what is the proliferation and differentiation rates in the different regions? Is there homeostasis?
2) Provide additional evidence that cells around the HF proliferate more during anagen.
3) Focus on the P42 data rather than the P21 data. Also here monitor the growth (if any) of the skin in their longitudinal experiments.
4) Carefully rewrite the document ensuring that titles, subtitles and experimental data and details is provided in the manuscript.

1st Revision - authors' response 20 July 2016

Many thanks for giving us the opportunity to revise our manuscript now entitled "*The bimodal evolution of clones in the interfollicular epidermis is regulated by hair follicles and their cycling*" (EMBOJ-2015-93806).

You will find below the reviewers comments (italicized) and our reply with additional experiments as discussed initially. I have also figured as comments the experiments that we proposed to perform in our initial rebuttal. This has resulted in a much stronger manuscript that now clearly demonstrates that keratinocyte clone size evolution is strongly dependent on hair follicle proximity and the hair follicle cycling. This adds to a handful of new theories in this field trying to understand whether surface epidermal homeostasis is related to stem cell activity or not. Our data brings new evidence suggesting that despite the existence of a hierarchy, progenitors identified by their quiescence have a context dependent activity.

As you will see, we believe all reviewers concerns have been addressed and certainly hope you will provide us with the opportunity to do so.

EMBOJ-2015-93806: Point by point reply and suggested experiments

Referee #1:

In this study the authors used a mouse model ("skinbow"), which allowed multicolor lineage tracing to investigate the clonal expansion of cells of the interfollicular epidermis (IFE). In particular, the authors aimed to examine the contribution of hair follicles and the hair renewal cycle to the turn over of the IFE specifically in back skin, which was not assessed in previous studies. The authors propose two modes of clonal expansion: 1) clones attached to hair follicles showed rapid increase in size during the growth phase of hair cycle; 2) clones distant from hair follicles were slow-cycling, but could be mobilized by proliferative stimuli. The "skinbow" mouse model could provide an interesting tool to study the clonal expansion of cells in the IFE of back skin. However, several claims are not supported by any data and the experimental design used in this study was often not suitable to convincingly prove the conclusions made by the authors.

There are major concerns and it is essential to address important issues to support the conclusions:

1) The authors used a tamoxifen-inducible mouse line and depending on the questions either a high or a low dose of tamoxifen was used. However, the authors do not provide all details and data necessary to support their claims and to demonstrate convincingly that the mouse model used in this study is suitable to address the proposed questions. The authors should provide further methodical details and present control experiments to exclude spontaneous recombination in the absence of tamoxifen, as claimed in the manuscript.

We agree with reviewer that these are important pre-requisites for the use and understanding of the model.
We have therefore added the following results"

a-We show clearly that in the absence of tamoxifen injection, there are no recombination occurring after observing mice for 12 weeks (FigS2a).

b-We show the staining efficiency of high and low dose tamoxifen as per our experimental conditions (Fig S2c).

c-Given the presence of 2 caggsbow construct in each cell, one could assume that cells that have only recombined one of the 2 construct (INCOMPLETE recombination resulting in orange or purple) have a theoretical possibility of recombining the second one resulting in a different colour (COMPLETE recombination resulting in either yellow, blue or white). We have compared the frequency of complete versus incomplete recombinations at early versus later time points and there is no indication that more complete recombinations occur later on suggestive of additional non-induced recombinations (Fig S2e).

2) The manuscript is lacking elementary information regarding labeling efficiency and specificity of the approach. Along the same lines, the first time points of analysis post recombination (3 weeks, 5 weeks) are too late since cell clones might already be lost from epidermis by that time providing a modified and wrong set of data subsequently altering outcome of the modeling.

We now provide labelling efficiency for high and low dose tamoxifen injection (Fig S2c).
We agree with reviewer that earlier time-point analysis will help better understand clonal dynamics. **Images of whole mounted skin at time-points from 2-5 days have been added to the manuscript in a separate figure**: this will allow visualization of clones prior to the full differentiation of the labelled basal layer cells (Fig S2b).

We believe however that the long term analysis also has its benefits and remains valid and strongly informative. Indeed after 3 to 5 weeks, as the referee indicates, clones emanating from differentiated and non-progenitor basal cells would have produced corneocytes that further shed away. We believe this provides some advantage as it allows the analysis to focus on the clonal fate of the actual cells that will help maintain the epidermis.

3) The authors injected mice either at P21 or p42 and aimed to label cells in growing mice after hair follicle formation was complete (P21) and in adult mice after the first hair cycle (P42). To convincingly show increased and rapid clonal expansion during the growth phase of hair follicles (anagen) it would be more important to label cells additionally in anagen phase (early anagen) and compare the clonal expansion during telogen and anagen. In most experiments, the authors harvested and analyzed the tissue 3 to 24 weeks after tamoxifen induction at P21 and P42. In the current setting, it is not clear how the authors distinguish between anagen and telogen hair follicles. The Wnt reporter is mentioned but no data are provided. The authors should choose more defined time points after tamoxifen induction, including time points allowing to systematically analyze initial labeling and clonal expansion.

We thank reviewer for this suggestion. We have now provided results from new experiments by inducing mice during anagen versus telogen. Animals were sacrificed after either 1 or 3 weeks post-induction. These new experiments strongly support our claim that clones attached to hair follicles in anagen expand more robustly (Fig 3).

4) The authors used FACS and confocal microscopy to study color distribution, number or size of clones. Using FACS alone, as done in Appendix Figure S2, seems to be an inaccurate way for quantification of the different clones per color, since there is no clear separation of the different populations, representing the different colors. The authors should provide an additional quantification using confocal microscopy.

In Fig S3c as highlighted by referee we provide quantification of colours using flow-cytometry but counts in FigS3d are performed using confocal microscopy. As requested we have now updated this figure with additional data showing the stability of colours obtained through recombination over time.

5) In the manuscript it is claimed, that small clones had cells in the basal layer of the epidermis in more than 80% of cases, with less than 20% being disconnected from the basal layer as expected in the case of clones undergoing terminal differentiation entirely and disappearing. The authors should support this conclusion by providing IF-co-staining for proliferation and differentiation marker.
In figure S4, we now provide evidence confocal images of our labelled cells co-stained with alpha-6 integrin a basal cell marker. This allows establishing clearly the existence of basal and suprabasal cells. This supports strongly our initial claim that more than 80% of small clones not attached to HF had cells in the basal layer.

6) Unfortunately, it is not clear how symmetric cell division versus asymmetric cell division was examined. The authors should provide details for this analysis and in addition use specific markers to examine spindle pool orientation during clonal expansion of labeled cells.

We apologize for the confusion around symmetric and asymmetric divisions. This is a misunderstanding due to poor choice of wording in the manuscript. In their original study, Clayton and colleagues described the three possible fates of a committed progenitor (CP) to wards either other CPs or post-mitotic cells (PM) that would enter terminal differentiation. CP cells could undergo a cell division towards 2 CPs, or towards 2 PMs at similar rates, or they could undergo a division towards 1CP and 1PM. All these divisions took place in the basal layer of the epidermis. We chose the unfortunate terms of symmetric divisions for both CP > 2CP and CP > 2PM, and coined CP > 1CP+1PM as asymmetric. However this definition is only based on the modelling performed in Clayton et al and does not preclude the orientiation of the spindle during cell division. We therefore apologize for the confusion and will change the wording of this paragraph.
More importantly we agree with referee that we have not provided enough details around our estimation of the proportions of respective fate choices in our model. This estimation was calculated in early time-point tracing of clones of 2-4 cells. Full detail disclosure of how this was performed is now added to the methods section of the manuscript (Supplemental methods file page 4).

7) The label retention experiments are not convincing. The authors should systematically analyze efficiency of initial labeling and provide quantification data for proliferation and quiescence of keratinocytes. It is not clear which time points post BrdU pulse were examined (Figure 5).
We would like to thank reviewer once again for pointing to these additional experiments. The initial experiment was performed after a 4 day pulse of BrdU and a chase of 5 weeks. This will be detailed in the text and the figure legend. We now provide a much more detailed label retaining experiment, strongly highlighting the initial efficiency of the staining across the IFE (multiple injections of BrdU over 72h). After this initial pulse, animals were sacrificed for analysis at various time-points (weekly up to 5 weeks) to show the progressive dilution of the BrdU label.
These new experiments strongly support our claim that label retention mostly occurs distant from the hair follicles.

8) To address the question if the detected slow-cycling cells respond to proliferative signals, the authors used TPA to stimulate proliferation and demonstrated a significant reduction in the proportion of small clones and a significant increase in large clones in TPA treated back skin. It would be interesting to demonstrate if the stimulation of proliferation induces more symmetric or asymmetric divisions. Furthermore, providing additional staining of a proliferation marker would be required to convincingly show that normally slow-cycling cells respond to TPA by increased proliferation and clonal expansion.

This is an exciting suggestion by reviewer, and will further prove that small slow cycling clones upon TPA stimulation enter proliferation. **TPA treated skin from skinbow mice injected with BrdU 2 hours prior to sacrifice was obtained. This now clearly shows that IFE distant from HF enters a very important proliferation (Fig 5).**

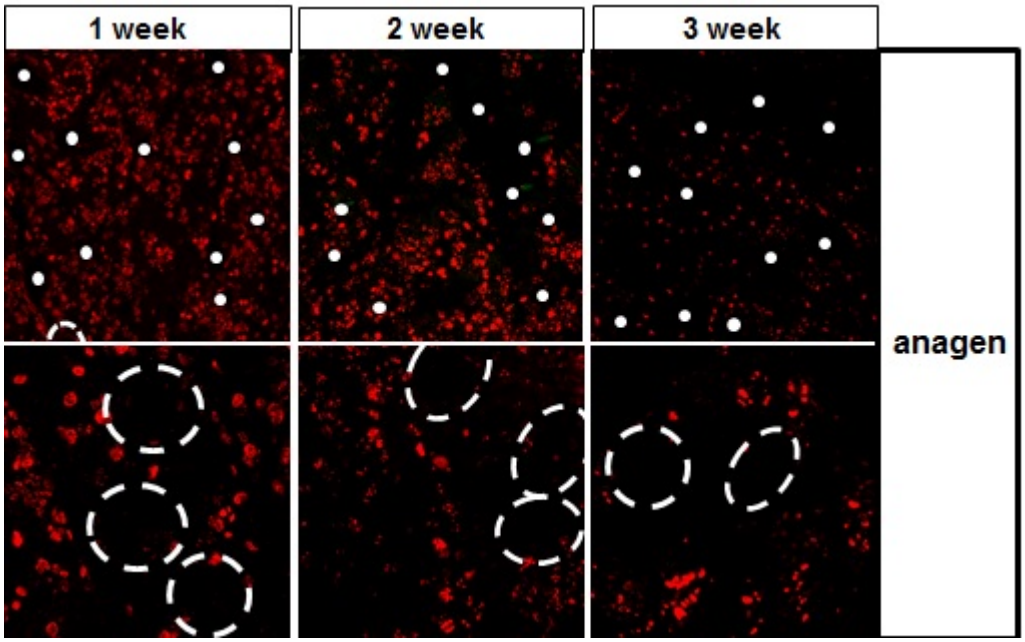
Regarding the symmetric or asymmetric division, we have no claim regarding the orientation of the spindle as detailed above and this was simply a poor choice of wording.

9) The authors propose an exchange between the two compartments of clones attached to hair follicle and clones not attached to hair follicles within three weeks. However the data provided here

do not support this hypothesis and are over-interpreted. Would it not be expected anyway that larger clones eventually attach to or grow close to hair follicles given the space restriction between hair follicles?

We have taken extreme care in evaluation the probability of a given clone to reach a hair follicle based on its size alone. Fig 2d simply indicates that larger clones are more frequently attached to HFs than what their size would theoretically predict. The methodology behind these models is fully detailed in supplemental material. We claim that clones attach to hair follicle will eventually distance themselves from HF and behave as slow-cycling clones (see model in fig 6d).
In figure 6 we present data suggesting that the proportions of clones attached and non-attached vary depending on whether mice are labelled at P21 or at P42. This clearly suggests that there are exchanges of clones between these 2 compartments.
A difficulty is to be able to track cells in proximity of hair follicles over time as no reliable marker or specific Cre has been identified for them. Based on our existing data, epidermal cells around hair follicle proliferate more intensely during anagen. We planned initially to use this using short term BrdU pulse experiments as a way to specifically label this population. However, BrdU was incorporated into cells around hair follicles but also other cells beyond. As can be seen below, cells labelled during a short pulse in anagen were further tracked after 1-3 weeks of chase showing clearly a growing distance between the hair follicle (dots in low magnification images and dashed circles in high magnification images) and the BrdU labelled cells (red). Although this supports our claim that over time clones attached to HF distance themselves from the hair, it could also be the result of the dilution of BrdU in proximity of hair follicles.

We have therefore toned down these conclusions in the manuscript as definite proof of these claims cannot be technically obtained.



In general, authors should carefully check that for each experiment the following information is provided: total dose/d; concentration of tamoxifen per ml; injection volume per kg mouse; number of injections and injection method (intra dermal or intraperitoneal). Furthermore, the authors should include the corresponding n-number of mice analyzed in figure legends. Finally, there seems to be a mistake in the graph of Figure 2 c (iii) and Figure 5 c: The bar representing large colony size (261 - over) is depicted differently within the graph itself and the figure legend.
In conclusion, the authors provide an interesting tool to study the clonal expansion of cells in the IFE of back skin. However, not all data are convincing and more data are required to support the author's conclusion. This study supports previous results published by other groups.
We apologize for this lack of detail. All requested information have been provided in the revised version.

Referee #2:

1) In the submitted manuscript, the authors assess cellular behavior in mouse back skin based on quantitative fate mapping. They perform their analysis from 2 time points (p21 and p42) and reach the conclusion that interfollicular epidermis is maintained by cells with 2 different behaviours depending on their location. Cells that are associated (attached to HF) to hair follicles appear to be more proliferative than cells distant from hair follicles (not attached to HF). By computer simulation using a stochastic lattice model, the authors predict that anagen follicles influence cells in their immediate neighborhood (10-30 basal cells) to proliferative faster (0.1-0.3/week compared to 3/week).

Understanding the mechanisms that control tissue homeostasis is important and the submitted manuscript goes a long way in describing this. There is however a number of problems with the manuscript that needs to be addressed.

The title states that 'Homeostasis of the interfollicular epidermis is regulated by hair follicle cycling'. Firstly, homeostasis describes a tissue in equilibrium where cell loss equals cell gain, yet most of the analysis and conclusions are derived from analysis of data, where animals are labeled before they reach adult size. There are significant differences between the 2 data sets (p21 vs p42) with much more prominent differences for the p21 dataset when assessing the differences between interfollicular epidermis that is either attached and non-attached hair follicles. Could expansion of the epidermis during mouse growth be driven by the areas closest to hair follicles?

We agree with reviewer that mice induced at P21 are still growing and that mice at P42 have a different profile of clone progression. Indeed Fig 6a in the manuscript strongly supports the fact that clones attached to HF contribute greatly to the expansion of the skin surface in mice labelled at P21. **In the revised version of the manuscript data focuses essentially on mice injected at P42** to reflect clonal dynamics during homeostasis. P21 data is used as supporting evidence.

2) Secondly, the authors state that the epidermis during anagen phase is proliferating more rapidly than during telogen. They show some images in figure 4 to support this notion. It is however unclear what the images represent.
A) Are these pictures obtained from adult animals?

Yes, animals used in Fig 4 are adult.

B) Why is the epidermis in early anagen multilayered as if the skin has been treated with TPA or another compound? C) Although the quantification appears solid the images provided to further substantiate the data (4A) are not representative of the quantification and there are no obvious Ki67 positive cells in the EMA, MA, C and T samples. The fractions should only be reduced.

In agreement with reviewer, we have now provided better images at higher magnification of the IFE at different phases of the hair cycle.

3) In addition, it is hard to appreciate from Fig4C that cells closest to the hair follicle are proliferating more than those further away as suggested by the clonal fate data and the LRC analysis.

Fig 4 C clearly shows a difference in proliferation (revealed by Ki67 staining) in the basal layer of the epidermis at early anagen versus telogen 5 weeks after labelling. It also shows that Ki67+ cells in anagen belong to the same clone (YFP+) attached to a hair follicle. Despite this existing evidence we have expanded these experiments as requested by reviewer. **We provide here BrdU labelling in a short 2 hours pulse during anagen as compared to telogen** that undoubtedly support our claim of more solid proliferation during anagen (Fig 4). Although the BrdU incorporation goes beyond the hair follicles, it clearly always includes the immediate surrounding of HF whereas other areas distant from the hair remain unlabelled.

More importantly, we now have short term labelling data where tamoxifen has been injected in telogen versus anagen. Seven days post-labelling, there is a marked increase in clone size in anagen. This is most obvious for clones attached to HF but occurs as well for clones distant from HF.

4) Moreover, if this is true the average clone size following the first anagen (w5 following p21 labeling) should be significantly greater in clones attached to HF compared to the non-attached ones (Figure 2a). The authors spend a lot of effort discussing the importance of anagen on clonal growth, but they fail to provide any explanation why the average size of HF attached clones increase 4 fold between weeks 5 and 12 following labeling. Moreover, the effect of time in the P42 data set appears less prominent. How does the p42 data look if plotted like the p21 data set in Fig 2a?

We thank reviewer 2 for pointing out these shortcomings. We have addressed all these points in the following :

a- Fig 2 a now provides the data for mice labelled at P42 as discussed in the first comment of reviewer 2 to reflect a homeostasis situation.
b- To overcome the difficulty regarding the effect of anagen on clone size, in figure 3, we have now provided an estimate of the number of hair cycle each region of the skin examined has undergone. This is reflected in the number of club hair present in the whole mount specimen analysed. Each additional hair club reflects an anagen phase in that epidermal region. Analysis of the data based on hair club number more clearly shows that clones attached to hair follicles grow based on the number of hair cycles whereas clones not attached to hair follicles remain of similar size (fig 3b)
c- As discussed above, when labelling is performed in adult mice during anagen versus during telogen, 7 days post labelling there is a strong and significant increase in clone size in anagen. This is mostly true for clones attached to hair follicles (Fig 3c).

5) The authors wish to show that the epidermis contains 2 distinct populations of cells characterized by different proliferation phenotypes. To address this they:
Firstly, perform a BrdU labeling experiment, where they inject animals repeatedly with BrdU at 21 days and chase for 5 weeks before analysis. There are multiple problems with these experiments since a cell will not take up BrdU unless it divides and they are therefore not likely to target very many of these cells. Moreover, BrdU is also killing fast cycling cells, which could affect tissue homeostasis. What does the tissue look like following 3 days of labeling and how many cells are actually labeled with BrdU? If cells far from the HF indeed are less proliferative a whole mount image as shown in figure 4c should illustrate this point nicely. To make solid conclusions of these observations, a thorough quantification should be completed. Moreover, the discussion of the data related to Merkel cells and touch domes is difficult to follow since there is no turnover in this compartment following development and BrdU should not label them.

We appreciate reviewer's suggestions that have now strongly improved figure 4. We provide now a series of whole mount images of BrdU labelling that highlight the following:

a- At 3 days after a pulse of multiple injections even in telogen it is possible to label most cells in the IFE

b- Images of BrdU labelled cells on a weekly basis up to 5 weeks strongly support our claim that the large majority of label retaining cells are distant from hair follicles. There is no labelling of Merkel cells in this new setting. In agreement with reviewer we have now retracted those initial claims.

6)Secondly, the authors perform TPA treatment and show that the fraction of small clones is reduced and state that this is evidence for their ability to contribute to homeostasis following injury. An alternative explanation is that a number of the small clones are lost during the TPA treatment leading to an apparent increase in the larger clones. This would fit with the observation that cells are streaming from the hair follicle into the interfollicular epidermis. Without loss of cells (clones) in the interfollicular epidermis, it is difficult to appreciate how the epidermis can accommodate the hair follicle influx. The experiment needs to be described in greater detail. Which mice are used? How old are they? Dose of TPA? Etc

As discussed in response to reviewer 1-point 8, **we have performed BrdU labelling** to definitely prove the important proliferation that occurs with TPA. It is unlikely that cells incorporating BrdU in multiple layers of the epidermis are in fact massively dying. Given the increase in the frequency

of large clones occurring in parallel to the reduction of small clones after TPA, the most likely scenario is that small quiescent clones are not disappearing but proliferating. Experimental details are now clarified in methods and figure legends as requested.

7)The authors need to carefully consider their subtitles, as a number of them are misleading. Multicolour lineage tracing for analysis of clonal population dynamics.

Are clones truly clonal? The authors also state that colour frequencies do not vary significantly over time. How has this been evaluated? Cerulean is increased >50%.

We have extensively expanded results and discussion on the possibility of clone overlap and how to take this into account. See point 10 below.
Regarding colour frequency, we counted the number of clones of a certain colour in each mouse at every time point. We hypothesized that given the random process of recombination, the frequencies of different colours should in average remain stable. The high increase noticed initially was probably due to the low number of experimental repeat. The existing data has been updated with additional experiments using confocal microscopy and shows the relative stability of different colours over time. The data points at 24 weeks are still reflecting 4 mice only which might explain the slight difference observed. It is however important to note that from one time point to the next different mice are being used and depending on the efficiency of the tamoxifen activity the colour percentages may vary. **See also reply to reviewer 1, point-4**.

8) Clone size evolution over time distinguishes two proliferation profiles.

The authors cannot state this based on their clonal fate data, where they observe some small clones and some bigger ones. The data is however very similar to that previously described by Jones and coworkers, and the authors go on to state that the clonal distribution is similar to that previously observed (Clayton et al., 2007, Nature), where the cells analyzed in tail epidermis could be modeled based on one particular cell behavior. How can the authors then conclude that small clone population arises from cells that are slow cycling?

We respectfully disagree with reviewer. Clayton et al reported data showing global increases of clone size over time. In this sense our data have similar trends that we have highlighted in the manuscript. However, in their data Clayton et al did not have any clone that would remain of a small size. ALL clones in Clayton et al increased in size over time. Our findings are further supported by the publication of Mascre et al 2012 and more recently by Sada et al (Nat Cell Biol. 2016 Jun;18(6):619-31.) where they found two modes of epidermal clonal evolution as described here. In both studies, a population of slow cycling clones existed in tail epidermis.

9) In the same section the authors state based on end point analysis that the some cell divide asymmetrically, whereas others divide symmetrically upon analysis of 2-4 cells clones. The authors cannot conclude that these are symmetric or asymmetric cell divisions giving rise to the outcome, as these end-point experiments relate to the final fate and not the outcome of the cell divisions. All the experimental data related to these experiments are missing (number of clones analyzed, distribution within the clones 2, 3 vs 4 cells, and the experimental details related to the timing)

We apologize for this lack of detail. The choice of wording (symmetric, asymmetric) is also unfortunate and has been changed in the revised version. Please refer to similar query from **reviewer 1 point 6**. As understood by the reviewer, we aimed at evaluating the final fate of each cell rather than symmetric versus asymmetric division. We have now clarified the methods for evaluation in the supplemental methods section.

10) Strategies to overcome clonal overlap

This is an odd title and is this really what the authors show? Firstly, it is unclear how a clone size > 7 cells at d3 is the only measure for merging 2 clones and qualifies clonal versus non-clonal. Some how the authors need to qualify their statement.

We apologize for the lack of clarity. In figure 1 we display the distribution of clone size at D3. As can be seen there is a continuum for clone size up to 4-5 cells but a pic of clone size superior to 7

cells. In 3 days, it is unlikely for an epidermal clone to reach such a size especially that these mice were injected during telogen at a time when the IFE is not highly proliferative. We have therefore argued that these clones of size >7 allow a good estimate of the proportion of clones with an overlap in colour. A similar strategy has been used in Mascre et al (nature 2012). This is now more detailed in the result section of the manuscript.

Secondly, it is unclear how the elimination of some larger clones will qualify the assumption that two populations of cells exist. The authors have essentially just removed a number of events with preference for clones of size greater than an arbitrary value. Based on the committed progenitor model, analysis of tissues in homeostasis (not p21) one would expect a continuum of clonal sizes as the authors also observe. I am therefore unsure whether the authors can use this data as evidence for a two-population model.

We apologize for the lack of clarity but respectfully insist that our results do not display a continuum in clone size but rather a dramatic proportion of clones that are of small size. (>60%) persisting at all time points subsequently. In Clayton's publication where a single population of committed progenitors is claimed to maintain the epidermis the proportion of clones of small size drastically decreased over time. In our analysis of clone size distribution we have never claimed to eliminate any clone according to its size. Finally these findings are once again very similar to reports by Mascre et al. (2012).

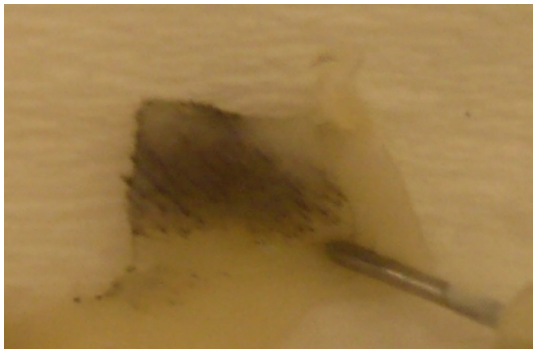
11) They go on to look at rare populations (white, pink and cerulean) in the same experimental data set. Whereas White and pink seems to behave similarly, it is unclear whether cerulean label is representative (EV2). It is enriched more than 1.5 fold after 12 weeks in the labeling experiments (S2d) and clonal distribution is significantly different in the data provided for the single colors. In order to prove or disprove the single progenitor model the authors need to address whether their data display the same scaling behavior as described (Clayton et al., 2007; Mascre et al., 2012; Doupe et al., 2010)

Lastly, they state that they perform an experiment with low dose tamoxifen, but the authors choose not to share the data and the evidence that their analysis is indeed performed at clonal densities.

We thank reviewer for these valuable suggestions. Low dose experiments were performed and displayed in Figure 1F and supplemental Fig 3. However, to alleviate all concern, we **have performed all comparisons across the rare colours** (anagen versus telogen and attached versus non-attached). Also as pointed in response to **reviewer 1 (point 1) we now provide full detail of recombination efficiency** in low-dose experiments.

12) The authors go on to describe clones as attached to anagen and telogen areas. The data in Figure 3 is however very difficult to interpret, as the entire back skin should be in telogen by day 56 (5 weeks) followed by sporadic anagen phase in an asynchronous manner induced randomly.

Areas in anagen were identified based on their pigmentation. Hair cycling after the completion of the first cycle is asynchronous. Although anagen is more likely expected around P70 on the entire back skin (Greco et al 2009), anagen waves can start more irregularly in different smaller areas of the back skin before this wave covers the entire back. We have furthermore extensive experience in quantifying this using Wnt bioluminescence reporters (J Invest Dermatol. 2014 Jun;134(6):1519-26). We agree therefore with reviewer that at P56 most of the back skin is in telogen but about 15% of it is in anagen and this will spread to occupy the entire backskin by P70. If required, data about bioluminescence images reflecting anagen entry between P42-P70 can be provided to support this claim. We provide here a macroscopic image of the anagen skin examined at P56.



As can be seen, these are pigmented areas in a small part of back skin. Further histology can be provided if needed.

13) The data for related to telogen versus anagen clones makes consequently little sense as this is just reflecting the current state of the epithelium and not necessarily how it has looked prior to the analysis. Moreover, the history related to whether one particular hair follicle has undergone single or multiple rounds of anagen is lost.

We respectfully disagree with reviewer. The entire back skin goes through the same number of hair cycle over time. In physiological situations, waves of anagen propagate throughout and usually do not spare any area. So when the entire back skin is in telogen, all areas have undergone the same number of hair cycles previously. Therefore comparing an anagen area to a telogen area FROM THE SAME MOUSE as performed in our experiments still makes sense as it allows reporting the effect of one additional anagen phase regardless of what has happened before.

We however agree with reviewer that having the information about the number of hair cycles that have occurred previously would allow us to compare different time-points or different mice. **As explained in point 4 above, we have now counted the number of club-hair** in each skin area as an indicator of the number of hair cycles in this area.

14) The authors describe growth of the organ by looking at distances between hair follicles (S4). How does this influence the shape of clones? Non-HF attached epidermis also expands more than HF attached? What about the size of the animal? Male versus female? How is this reflected in the lattice model? According to the measurements the mouse never reach homeostasis at least based on the structure of the unit measured. From which part of the mouse has these measurements been obtained?

Although these are interesting points raised by reviewer, we feel they are beyond the main objectives of our study. The shape of clones has seemed random to us and we have not evaluated it further. We have not performed association with animals' size and both males and females have been used in experiments. Of note, these measurements have been obtained from the mouse back skin and mostly reflect the growth of mice injected at P21. For consistency we **provide similar measurements for animals induced at P42 that demonstrate the stability of this distance over time for the duration of the experiment.**

The data related to the data simulation in EV5 is very difficult to comprehend as none of the simulations taking into account hair-follicle attachment (21 and 49) compared to no HF seems to be quantitatively better than the others. Some quantifiable measure for how one model is better than the other is required. Also, the similarity between experimental and modeled probabilities in EV5b is not that convincing. What does the model actually show? Something in between or that none of the proposed suggestions are correct.

Fig 6b and 6c show that if the final model doesn't take into account a differential behaviour of committed progenitors in proximity of hair follicles, it diverges from the experimental observations. Supplemental figure EV5 shows the effects of altering the definition of proximity to hair follicles (21 cells or 49 cells). EV5a shows that the experimental clone size distribution is well fitted with the model either without hair follicles, or when considering a hair follicle proximity of 21 cells, however when the proximity is increased to 49 cells the fit to experimental data deteriorates

significantly at the later time points (12 and 24 weeks). We quantified this by calculating the root-mean-square of the deviation between experimental data and the different simulation scenarios:

$$\sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(data_i^{exp} - data_i^{model}\right)^2}$$

	No HF	HF region 21 cells	HF region 49 cells
3 weeks	0.0159	0.0114	0.0127
5 weeks	0.0220	0.0193	0.0248
12 weeks	0.0151	0.0139	0.0224
24 weeks	0.0164	0.0155	0.0233

From this it is clear that for all time points a model with a HF delimited to 21 cells surrounding it has the smallest deviation from the observation. This table is now added to supplemental results to support data in EV5a.

EV5b shows the same as Fig6b, i.e. that the spatial correlation of clone sizes relative to hair follicles is not consistent with the model that does not take into account the activation around the hair follicles, however the fit to experimental data cannot be improved further by increasing the proximity where activation occur to 49 cells (the data for 21 or 49 cells almost overlap). Thus, from these simulations, taking into account both clone size distribution (EV5a) and spatial clone size correlation (EV5b) the size of the region influenced by the activity of hair follicles is approx. 20-30 cells.

Minor comments:
Page 3: Jones et al., 1995 never analyzed cells isolated from tissues but based their entire analysis on cultured keratinocytes
This reference has now been removed.

Figure 2A: The line connecting the values need to be removed and the data could potentially benefit from introduction of a log2 scale.
This figure has been amended with data from P42.

It will be helpful if the authors describe in detail what they measure. What does a clone of > 30 cells represent? Is this basal and suprabasal cells
The clone sizes represent basal and suprabasal cells.

2nd Editorial Decision 16 September 2016

Thank you for the submission of the revised version of our manuscript (EMBOJ-2016-93806R) to The EMBO Journal and my apologies again for the delay in processing this manuscript, which was caused by delayed re-review due to the summer time and travel commitments. Your revised manuscript has now been seen by the two original referees, whose comments are enclosed below.

As you will see, the first referee and had remaining concerns on the technical quality of the study and was much more critical than the second, however after cross-commenting, we decided - in light of the strong support of the latter - to give you the opportunity to revise your manuscript to address the referees' points. Thus, I would like to invite you to submit a final revised version of the manuscript using the link enclosed below, addressing the reviewers' comments.

As you will see, referee #2 finds that his/her concerns have been sufficiently addressed and is in favour of publication. However, reviewer #1 remains more critical and expresses remaining reservations related to incomplete experimental documentation that in his/her view weakens the robustness of the study. Accordingly, this referee asks you to strengthen the current conclusions by carefully revising the technical documentation. Please note that in addition to the original reports, referee #2 has also provided extensive cross-comments on the issues raised by referee #1, arguing in favor of the current manuscript, e.g. with respect to potential spontaneous recombination (ref#1, pt.

1) '...The authors show in their supplemental material S3a that they observe no spontaneous recombination without tamoxifen in 12w old animals. Leakiness is of course a concern in all studies related to fate mapping studies relying on inducible cre lines, but even if a small number of these events, going against the statement from the authors, should arise from leakiness it is not going to change the main message of the manuscript. ' , the issue raised on initial labeling (ref#1, pt.3) '...The authors have throughout the document except for 3c indicated the number of clones analysed, which tend to be much greater or at least on par with other studies analysing cell behaviour using lineage tracing.' or the classification scheme used (ref#1, pt.3d) '...The classification based on the number of club hairs is very innovative and much more precise than based on the staging. Small differences within the epithelium could interfere with the analysis. Moreover, the analysis based on club hairs, which could potentially underestimate the number of hair cycles if club hairs are lost following anagen. The analysis is consequently appropriate. ' .

Please note also that we conclude that more mechanistic data on the signaling involved as suggested by referee #1 is certainly of interest, but in our view beyond the scope of the current study.

Considering all of these points, we invite you to submit a revised manuscript addressing referee #1' comments. Along these lines, I would like to ask you in particular to

• provide additional control data on the TPA study or adjust the conclusions made (ref#1, pt.8)
• re-consider the claims regarding exchange of clones between the two compartments (ref#1, pt. 9)
• revise and complement Figure 5e (Ref #1, add. pt. (D))

Importantly, I ask you to integrate and discuss the recent related findings published by the A. Sada et al. (PMID:2718341) e.g. in the discussion.

Please see below for some formal formatting issues, which need to be adjusted at re-submission.

Please contact me if you have any questions, need further input on the referee comments or if you consider engaging in a compelling revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

(Report for Author)
In the revised version of the manuscript entitled "The bimodal evolution of clones in the interfollicular epidermis is regulated by hair follicles and their cycling" the authors propose two modes of progression of individual keratinocyte clones within the interfollicular epidermis (IFE) of mammalian skin and show the impact of hair follicles and the hair follicle regeneration on clonal behaviour of IFE cells.

The authors aimed to address the critical comments raised previously and the manuscript has clearly improved; in particular experimental details are now better explained.

However, some of the initial and most critical concerns remain and the main conclusions are still not sufficiently supported by experimental data. The quality and precision of individual experiments still require improvement.

Responses to major concerns as addressed by the authors:

1. Some technical concerns regarding the mouse model still remain. In addition to new data included in appendix S3a more quantitative analysis of recombination events is required (e.g. FACS for different colour combination of reporter) to convincingly exclude spontaneous recombination.
One reason for the concern is provided in Figure 3c: according to the figure legend the data are shown for post labelling day 7. However, the size of clones after 7 days (5%, 10% of clones 131-200

and 31-130 cells) indicate unspecific labelling prior tamoxifen application.
minor point in Figure 1b: pattern of bars need correction

2. Minor point: Fig S3 (not S2c)

3. Furthermore, the important issue regarding the initial labelling has not been addressed. Although the authors included data on labelling efficiency with high and low does of tamoxifen and Cre recombination, no information on the number and the localisation of these labeled clones is given. Since one of the main conclusions refers to the size and the growth rate of clones attached to hair follicles, it is absolutely required to provide these essential information.

3b. Moreover, it is not clear on what basis authors estimate demarcation of individual clones following tracing. Why it is excluded that more than one cell gives rise to the bigger clones? An example is given in Figure 1a where it seems difficult to determine how many clones are actually shown.

3c. The authors did not provide information regarding the Wnt reporter mice as requested.

3d. Thus, the classification into anagen and telogen stage is still not convincing. Given synchronisation of the hair cycling, it is recommended to analyse the epidermis at distinct time points and hair cycle stages.

3e. There are no hair follicles shown to prove stages. The statement that skin appeared grey or black is not proving anagen stage since in non-synchronised skin hair follicles will be in different stages (could be anything from early, middle, end of anagen and catagen) and could also contain telogen hair follicles.

4. Point has now been addressed in revised manuscript.

5. The EPU model is not discussed although experimental data indicate stem cell-driven formation of columns in IFE.

6. Point has now been addressed in revised manuscript.

7. The quantification of LRC is required in Figure 5a to support the conclusions.

8. The TPA study (to demonstrate proliferation of smaller clones) is lacking control experiments and in its present form do not support the main conclusion.

8b. Figure 5d does not show stacks of clones as claimed in the manuscript.

9. The authors state "clones attached to hair follicles will eventually distance themselves from hair follicles and behave as slow cycling clones". There is no experimental evidence to support this conclusion.

The reviewer does not agree with the statement "there are exchanges of clones between the two compartments" since the data are based on comparing clones labelled at different time points at different stages of the hair cycle. Thus, this comparison seems not valid and does not allow any conclusion.

Taken together, the paper has potential and the initial observation of clones with different growth modes is very interesting.

However, the manuscript is too descriptive and quite lengthy (especially the first part contains redundant observations). The modelling experiments are difficult to understand and are not well explained within the result chapter.

The manuscript would greatly benefit from more mechanistical data. Perhaps the authors could discuss which signal from hair follicles (associated with anagen) could be responsible for increased growth rate of clones attached to hair follicles.

Additional comments:

(A) Figure 4a: the quality of images needs improvement, no hair follicles and Wnt reporter are shown.
(B) Figure 4c: overexposure of reporter-positive clones (yellow).
According to the images provided, the proliferation rate is not higher in cells close to hair follicles as claimed by the authors.
(C) Figure 5e: parts of the figure are missing.
(D) It is not clear why the authors come to the conclusion of the committed progenitor (CP) model given that the experimental data do not support this statement (e.g. BrdU/EdU tracing not convincing and no quantification of these results)

Referee #2

(Report for Author)
I commend the authors for their work leading to the revised manuscript. The authors have carefully addressed all my major concerns and I fully support publication of the manuscript. This is extremely timely as it sheds light on important aspects, which have not been addressed in a recently published paper in Nature Cell Biology (Asda et al., 2016).

2nd Revision - authors' response 23 September 2016

Many thanks for giving us the opportunity to submit a revised manuscript (EMBOJ-2016-93806R) upon second review and for your suggestions to improve the manuscript. We have here enclosed our reply to the comments you and reviewers raised and have accordingly modified the manuscript. We certainly hope you will find this revised version suitable for publication.

Editor's comments:

...Considering all of these points, we invite you to submit a revised manuscript addressing referee #1' comments. Along these lines, I would like to ask you in particular to

• *provide additional control data on the TPA study or adjust the conclusions made (ref#1, pt.8)*
We apologize for this omission and have now added additional panels to Figure 5C, clearly demonstrating the lack of proliferation as based on Ki67 expression on the same mouse in areas where TPA has not been applied. This ensures the quality of comparisons made in figure 5E and 5F.

• *re-consider the claims regarding exchange of clones between the two compartments (ref#1, pt. 9). Importantly, I ask you to integrate and discuss the recent related findings published by the A. Sada et al. (PMID:27183471) e.g. in the discussion.*

We agree with Editor and reviewer 1 that this is a contentious point. We have now removed this claim from the results and have discussed it as a possibility supported by recent data published by Sada et al.

Please see below for some formal formatting issues, which need to be adjusted at re-submission.
We have also proceeded with the corrections of the formatting issues.

Referee #1

(Report for Author)
In the revised version of the manuscript entitled "The bimodal evolution of clones in the interfollicular epidermis is regulated by hair follicles and their cycling" the authors propose two

modes of progression of individual keratinocyte clones within the interfollicular epidermis (IFE) of mammalian skin and show the impact of hair follicles and the hair follicle regeneration on clonal behaviour of IFE cells.

The authors aimed to address the critical comments raised previously and the manuscript has clearly improved; in particular experimental details are now better explained.

However, some of the initial and most critical concerns remain and the main conclusions are still not sufficiently supported by experimental data. The quality and precision of individual experiments still require improvement.

Responses to major concerns as addressed by the authors:

1. Some technical concerns regarding the mouse model still remain. In addition to new data included in appendix S3a more quantitative analysis of recombination events is required (e.g. FACS for different colour combination of reporter) to convincingly exclude spontaneous recombination. One reason for the concern is provided in Figure 3c: according to the figure legend the data are shown for post labelling day 7. However, the size of clones after 7 days (5%, 10% of clones 131-200 and 31-130 cells) indicate unspecific labelling prior tamoxifen application.
minor point in Figure 1b: pattern of bars need correction

We understand reviewer's concern about spontaneous recombinations. However we've brought clear evidence of their absence at 12 weeks in the absence of tamoxifen injection. The size of clones in figure 3c are quite large as noted by reviewer. However this is only in areas of anagen where we propose that IFE epidermal cells largely proliferate. Even at a rate of one cell division per 24h, 7 cycles of divisions results in 128 cells. Therefore the findings reported are not incompatible with our claims as suggested.
Observation of spontaneous recombinations if they exist are therefore a very rare event that we have never been able to observe. Such rare event is unlikely to influence our analysis based on sizes of hundreds of clones.

2. Minor point: Fig S3 (not S2c)

We have verified the order of figures and supplemental figures in the main text.

3. Furthermore, the important issue regarding the initial labelling has not been addressed. Although the authors included data on labelling efficiency with high and low does of tamoxifen and Cre recombination, no information on the number and the localisation of these labeled clones is given. Since one of the main conclusions refers to the size and the growth rate of clones attached to hair follicles, it is absolutely required to provide these essential information.

This is an important question. The recombination was randomly distributed across the IFE as illustrated in Appendix Figure 3b and 3c for high and low density staining. This is now clarified in the result section of the revised manuscript (page 6, last paragraph)

3b. Moreover, it is not clear on what basis authors estimate demarcation of individual clones following tracing. Why it is excluded that more than one cell gives rise to the bigger clones? An example is given in Figure 1a where it seems difficult to determine how many clones are actually shown.

We have provided four different lines of evidence to demonstrate that our data is representative of clones. These are detailed in the result paragraph strategies to refine clone size estimates. Among these methods, the use of low density staining and the use of rare colour recombination clearly rule out a major confounding role of clonal overlap in our data.

3c. The authors did not provide information regarding the Wnt reporter mice as requested.

We apologize for the confusion. The Wnt reporter mouse was used to source the skin samples from different phases of the hair follicle cycle that were used to produce figure 4A. The model and the evaluation methods of the hair cycle are validated and published (reference Hodgson SS et al, PMID 24531689 from manuscript).

3d. Thus, the classification into anagen and telogen stage is still not convincing. Given synchronisation of the hair cycling, it is recommended to analyse the epidermis at distinct time points and hair cycle stages.

We respectfully disagree with reviewer. Induction at different time points is likely to introduce bias regarding staining efficiency. In all our comparisons we have obtained anagen and telogen areas from the same animal.

3e. There are no hair follicles shown to prove stages. The statement that skin appeared grey or black is not proving anagen stage since in non-synchronised skin hair follicles will be in different stages (could be anything from early, middle, end of anagen and catagen) and could also contain telogen hair follicles.

In the previous revision we provided reviewer with images of the skin whole mount showing both anagen and telogen areas. In mice even after the first cycle of synchronised anagen, hair follicle cycles progresses in waves of anagen affecting domains in the skin where ALL hairs remain in phase. This is amply supported by literature (Plikus et al, Nature 2008; or from our own group as cited above: Hodgson et al PMID 24531689). Non-synchronized skin occurs after the first cycles and results in differnet domains being in different phases of the cycle. However within a given domain all hairs are synchrone and in the same phase.

Secondly, to alleviate all concern we provided in Fig3b the evolution of clone size according to the number of club hair reflective of past anagen phases. In this analysis, the presence of past anagen is undeniable. This analysis clearly supports the rest of our data suggesting increased clonal size in anagen phase.

4. Point has now been addressed in revised manuscript.

ok

5. The EPU model is not discussed although experimental data indicate stem cell-driven formation of columns in IFE.

We have now discussed the EPU model in the revised discussion.

6. Point has now been addressed in revised manuscript.

ok

7. The quantification of LRC is required in Figure 5a to support the conclusions.

Figure 5a demonstrates clearly that immediately after BrdU labelling, many cells around hair follicles are stained. Over time, there is indeniably a clear gap with no BrdU positive cells along the chase period. This is detailed in the results section page 13, 3rd paragraph). A quantification of the number of LRCs as requested by reviewer will simply show their reduction in number which is not the main point of this experiment. We therefore respectfully suggest that figure 5a remains unchanged.

8. The TPA study (to demonstrate proliferation of smaller clones) is lacking control experiments and in its present form do not support the main conclusion.

We apologize for this omission. Fig5c now contains control skin from areas from the same mice where TPA has not been applied showing a lack of epidermal proliferation in the telogen

IFE in the absence of TPA as expected.

8b. Figure 5d does not show stacks of clones as claimed in the manuscript.
Figure 5d is a stack of images of the same skin region from the stratum corneum down to the dermis.

9. The authors state "clones attached to hair follicles will eventually distance themselves from hair follicles and behave as slow cycling clones". There is no experimental evidence to support this conclusion.
The reviewer does not agree with the statement "there are exchanges of clones between the two compartments" since the data are based on comparing clones labelled at different time points at different stages of the hair cycle. Thus, this comparison seems not valid and does not allow any conclusion.

We understand and share reviewer's concerns. We had toned down our conclusions on this topic but in agreement with reviewer we have now removed all claims regarding the exchange of clones between the two compartments.

Taken together, the paper has potential and the initial observation of clones with different growth modes is very interesting. However, the manuscript is too descriptive and quite lengthy (especially the first part contains redundant observations). The modelling experiments are difficult to understand and are not well explained within the result chapter.

The manuscript would greatly benefit from more mechanistical data. Perhaps the authors could discuss which signal from hair follicles (associated with anagen) could be responsible for increased growth rate of clones attached to hair follicles.

Although of interest, this represents a major undertaking that may lie beyond the aims of the present study.

Additional comments:
(A) Figure 4a: the quality of images needs improvement, no hair follicles and Wnt reporter are shown.
(B) Figure 4c: overexposure of reporter-positive clones (yellow).
According to the images provided, the proliferation rate is not higher in cells close to hair follicles as claimed by the authors.

As discussed above, the Wnt reporter has been described previously and was only used in Fig4a. **High quality images have been prepared and will be provided in the final submission.**

(C) Figure 5e: parts of the figure are missing.
Fig5e is complete, the right part of this panel is a mask of small clones only.

(D) It is not clear why the authors come to the conclusion of the committed progenitor (CP) model given that the experimental data do not support this statement (e.g. BrdU/EdU tracing not convincing and no quantification of these results)

This is now better summarized in the discussion.

Referee #2

(Report for Author)
I commend the authors for their work leading to the revised manuscript. The authors have carefully addressed all my major concerns and I fully support publication of the manuscript. This is extremely timely as it sheds light on important aspects, which have not been addressed in a recently published paper in Nature Cell Biology (Asda et al., 2016).

Many thanks. This additional reference is now discussed in support of our model.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-93806

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "Analysis"
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "Analysis"
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "the skinbow model"
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "Analysis"
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "Analysis"
5. For every figure, are statistical tests justified as appropriate?	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "Analysis"
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "Analysis"
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Ki 67: Supplementary text, section "Immunofluorescence" Lrig1: Supplementary text, section "Immunofluorescence" BrdU: Supplementary text, section "Bromodeoxyuridine (BrdU) immuno-staining"
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	EMBOJ-2016-93806-AppendixSupplementaryMethods, section "the skinbow model"
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Main text, section Material and Methods, "Mice" paragraph
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The ARRIVE guidelines have been consulted.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA