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TCF/Lef1 Activity Controls Establishment of Diverse Stem and Progenitor Compartments in Mouse Epidermis

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(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 21 March 2011

Thank you very much for submitting your research study for consideration to The EMBO Journal. The comments from three expert scientists are overall encouraging, but demand some additional experimental work to improve the overall significance of your findings and that will have to be added during revisions.

As most of the comments from refs#1 and #3 are rather straightforward, there is not much need to repeat them here in full. Bottom line is that inclusion of bulge marker and cellular quantifications should enable reaching more definitive conclusions. Little more critical are the comments from ref#2 who would despite cell counts would prefer at least some 3D-reconstructions to resolve the issue of origin and lineage commitment in mouse epidermis. S/he also has ample of advice on the order of data representation to form a more coherent story that should overall focus on discussing the cell fate of normal follicles.

All in all, I kindly ask you to address these major critiques before returning a revised version of your manuscript for final scientific assessment.

I do have to remind you that it is EMBO Journal policy to allow a single round of revisions only and that the final decision on the study still depends on the content and strength of the revised version.

In case of further questions, please to not hesitate to contact me, preferably by E-mail.

Thank you for the opportunity to consider your work for publication. I am very much looking forward to your revision.

Yours sincerely,

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Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this manuscript, the authors analyze the contribution of bulge cells to multiple differentiated progeny of pilosebaceous unit. In particular, they focus on the contribution of bulge cells to the sebaceous gland. To do so, they cross a mouse line expressing a tamoxifen inducible cre recombinase under the bulge specific K15 promoter with reporter mouse lines. Lineage tracing analysis reveals that bulge cell progeny contribute to the renewal of the sebaceous gland during skin homeostasis independent of the hair cycle stage. Through careful analysis of gene expression and the time-course of bulge cell progeny out of the CD34 region of the bulge, the authors demonstrate how bulge cells progress through multiple regions of the follicle as they exit the stem cell niche. In addition, the authors provide strong evidence that expression of the dominant active NLef1 transgene results in the contribution of bulge cell progeny to ectopic sebaceous gland formation. This manuscript addresses several important concepts that are important for the stem cell and skin field, including the relationship of stem cells to their progeny and Wnt signaling contributes to progenitor formation. In general, the manuscript is well written and the experimental data presented are convincing. The in vivo imaging experiments, careful lineage tracing, and various mouse models utilized by the authors provide innovative and informative data regarding the activity of stem cells in the skin. In some cases, the conclusions are not fully supported by the data presented. With additional data and textual changes, this manuscript will be appropriate for the EMBO Journal. Specific comments are below.

1. For both reporter lines, the authors should perform a detailed analysis of labeling after 3 days of induction to validate the absence of reporter expression in the basal epidermis throughout the entire back skin and within the tail upon tamoxifen treatment.

1. Figure 1. a. It would be useful to know how many cells are labeled at each timepoint. Is there an increase in the number of cells labeled at each timepoint? This would indicate that the cells are proliferating during their transition to the sebaceous gland.

b. Do labeled cells contribute to the hair germ during this experiment?

c. The authors state throughout the manuscript that cells are seen within the sebaceous gland duct. How is this visualized? Examples of this labeling are not evident from the data presented.

d. The labeling in Figure 1 is referred to as "single" bulge cells. Because single refers to one cell, perhaps individual would be a better term.

e. The reference to the control data in Supplemental Figure 2 should be moved to after the first paragraph in the results section before the discussion of the lineage tracing results.

2. a. The authors conclude from the data within figure 2 that "labeled bulge cells are able to replenish the HF SC pool over long periods in adult skin and this process seems to involve symmetric cell division". The use of "symmetric" cell division is not clear: are you referring to the axis/direction of division or the differential cell fate of stem cells to self-renewal or differentiation? This conclusion may be based on the in vivo lineage tracing experiment in Figure 5. Further analysis of these live imaging data (quantification of the direction of cell division) or clarification by what symmetric division means is required. Furthermore, quantification of the number of cells labeled in the bulge over time (see points above for Figure 1) may make the "symmetric cell division" statement more clear.

b. The authors conclude that their data "argues against the possibility that genetically labeled progenitor cells are residing within and regenerate the SG. Instead the results prove that HF SCs are an important source for constant renewal of SGs". However, this conclusion is too strong based on the data presented. Since SG progenitor cells that are replenished by bulge cells would be genetically labeled with YFP or lacZ marker, the contribution of bulge cells vs progenitor cells in a long-term labeling experiment cannot distinguish between bulge cell or progenitor cell contribution. Furthermore, the data in Figure 5D demonstrate that a previously identified marker of SG progenitor cells (Blimp1) is upregulated in labeled cells after 6 days.

3. Figure 3. a. The data in Figure 3 could be improved with the use of a bulge cell marker. Given the abnormal appearance of the follicles in K14- NLef1 mice, the location of the bulge is not evident.

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b. Quantification of these data similar to that performed in Figure 1 in WT mice is needed. Additionally, the number of cells in each region of the follicle (bulge, hair germ vs sebaceous gland) should be quantified for each timepoint.

4. Figure 4. Quantification of the number of cells in each cell location (CD34/LRC/K15, MTS24, Lrig1 vs SCD1) at each timepoint should be performed.

5. Figure 5. a. The data in Figure 5A may be more appropriate within Figure 1 or Figure 2. Additionally, the quantification of these data in Figure S5a should be included within the main manuscript.
b. The real time PCR data in Figure 5C and 5D is not clear. Do the labels on the X axis indicate sorted cell populations? If so, the use of multiple markers should be used (alpha 6 integrin and CD34; MTS24 and alpha 6 integrin). The fold increase D5/D2 would be more informative regarding how gene expression changes in YFP+ cells over time.

6. Figure 6. The source of material for analysis of genes expressed by bulge cells or non-bulge cells is not clear. Sorted alpha6 integrin+, YFP+ cells should be analyzed in this experiment.

7. Figure 7. The K15- NLef1 transgenic mouse model provides strong evidence that the bulge cells contribute to ectopic SG formation. The CD34+ cells near the sebaceous gland are not clear in Figure 7F. Furthermore, the significance of CD34+ cells near the gland is not clear either. Are other bulge SC markers expressed in this location?

8. Abstract. The data presented do not support the conclusion within the abstract that "this process of de novo SG formation involves the establishment of new progenitor niches". Furthermore, how these data show "the recapitulation of early steps of tissue morphogenesis" is not clear.

Minor points:
1. The SD abbreviation is confusing. SG duct would be less confusing.
2. The verb usage for progeny is singular and should be plural. (see page 8, first paragraph).
3. On page 8, first paragraph, the following sentence should be edited: "frequency of labeling of bulge cells was lower in the back when compared to tail epidermis" to "frequency of labeling of bulge cells was lower in the follicles within the backskin when compared to those in the tail epidermis".
4. The text used to label axes of the graphs in the Figures should be increased.
5. Fig S1 is missing subheadings (A, B, C...)

Referee #2:

This study sets out to investigate cell fate and lineage in the hair follicle and sebaceous gland (SG) in transgenic mouse epidermis and how this is altered by an activated wnt pathway mutant.
The introduction highlights the two current models of SG homeostasis, ie that they are maintained by a dedicated SG progenitor population or by the proliferation of stem cells in the hair follicle bulge.
The complexity of hair follicle stem cell markers can be bewildering to a non specialist and it would be worth including a diagram of the follicle to summarize the different cell populations and their location.
The study begins with the generation of new transgenic lines using the keratin15 promoter to drive the expression of an inducible cre. One line labels most of the follicle bulge whilst a low expressing line permits cell labeling at clonal density, providing a powerful tool for the authors to investigate bulge cell fate. Reporter expression is analyzed in wholemount which is a real strength, and both bgal and YFP reporters are shown to give consistent results. It would be helpful to state how many hair follicles were scored for YFP with the uninduced K15 low strain animals, as a lack of background recombination in SG is crucial for interpretation of these experiments.
The fate of cells following low level induction is then tracked. 3 days after induction, 90% of the labeled cells are located within the bulge, with the remainder in the sebaceous gland or "complex", ie in the bulge, sebaceous gland and sebaceous duct. The proportion of complex hair follicles rises to 40% at 8 days. This data is interesting but would be strengthened by counting the size of the

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groups of labeled cells, which should represent clones if single cells are present at the start of the experiment. Crucially are multicellular clones which extend from the bulge to the SG seen at day 8? This may be the case in Fig 1I, for example, but it would help to show appropriate views from 3D reconstructed Z stacks to clarify this issue. In the current presentation the data do not "clearly indicate that the HF bulge contains a population of multipotent SCs contributing to the renewal of the SG during skin homeostasis."
The characterisation of the cells targeted by the K15 (suppl fig 2) might be better moved before figure 1.
The discussion about clones in different phases of the hair cycle and the presence of bulge derived cells (Suppl Fig 3) would be strengthened by 3D views showing contiguous clones extending from the bulge at a sufficient magnification to be clear to the reader.
In the long term labeling studies, Fig2, there is an interesting increase in the number of cells/bulge. If continual replacement of SG cells by bulge progeny is occurring, there should be clones extending from the bulge into each labeled SG at 180 days. It is not clear from the images presented if this is the case. How does the size of the bulge derived clones compare with that at 3 and 8 days?
The presence of clones with no connection to the bulge at 180 days argues that the SG contains a self maintaining population of progenitors. This is an important observation that should be discussed. Again it would be helpful to show images of sufficient quality to convince the reader that these clones have no connection with their size and compare the size distribution of these clones with that at early time points.
The authors then move on to investigate the lineage tracing in mice expressing a dominant negative Lef1 transcription factor. Bulge derived cells contribute to the ectopic SGs induced by expression of the mutant (fig 3). It would be interesting to know if labelled SG clones with no connection with the bulge persist at the 90 day time point.

The expression of markers in labelled cells is then investigated. The low power of the images in Fig 4 makes it difficult for the reader to confirm that individual labelled cells do indeed express the markers shown, some higher power insets which avoid oversaturation of the green channel would help here. Why is the FACS data included in 5B-D not presented as part of Fig 4?
Fig 5 A and the associated supplementary Fig 5 are a highly impressive experiment using time lapse imaging to track cell fate in explants. This should be highlighted in the main text and deserves a main figure on its own. The data should be presented in more detail and discussed more fully. In the current version the impact of the live imaging is lost and it is unclear why it is placed in the middle of a discussion about markers. Minor point: The same paper is Lu 2006 in the text and Lu 2007 in the reference list.
Fig 6 is a molecular characterisation of cells in the mutant phenotype. Given the complexity of the system the interpretation of the significance of the multiple markers examined is more speculative (the mutant may alter the expression of these genes independently of changes in cell behaviour) . It is unclear what this level of analysis of the "neomorphic" mutant phenotype adds to what is already a strong story about cell fate in normal homeostasis. Similar comments apply to Fig7, which shows the effect of expressing the mutant from a K15 promoter.

In the discussion citing the Langton 2008 paper seems inappropriate, as this shows that epidermis heals without input from hair follicles in Edar pathway mutant mice.
Overall there is some very interesting data here. The authors could do a better job of presenting this however, and given the limited space available in a single paper, might perhaps focus more on normal homeostasis than the "artefactual" generation of SG by transgenic expression of a mutant protein. The paper hinges around 3D imaging of clones, and would be strengthened by 3D rendering of Z stacks to illustrate the key points about the clones: the current images are difficult to interpret and are often at too low a power to assess the two color immunofluorescence. The standard of English is poor, with multiple spelling and grammatical errors throughout. I would urge the authors to revise the text with the help of someone fluent in English. If the issues discusses are addressed I would support publication in the EMBO Journal.

Referee #3:

In this work, Peterson et al have addressed the contribution of hair follicle stem cells to the different compartments of the pilosebaceous units, and show that bulge cells contribute to the turnover of SGs

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and the stem cell niches within the isthmus region. The specific contribution of bulge cells to the different compartments of the hair follicle, and their dynamic behavior, is still under in the field. Previous works have suggested that the bulge contributes to the turn-over of the SGs, however, how this occurs, and whether this is dependent on a specific phase of the HF had not been studied in detail.

The authors here provide compelling and elegant evidence for the complex dynamic behavior of the bulge stem cells, showing how and when they contribute to the SG and to the isthmus regions. Their in vivo time-lapse imaging is simply superb, and to my knowledge will be the clearest and most unequivocal evidence provided so far in the field for a direct contribution (i.e. migration) of bulge stem cells to the SG and UI. In addition, in it is still under debate whether bulge stem cells divide symmetrically or asymmetrically. Whereas this might be clearer for the contribution of bulge cells to the HF growth per se, the authors now convincingly show that the bulge cells proliferate symmetrically to subsequently migrate towards the SG progenitor niches for contributing to SG renewal.

I only have one general criticism to the manuscript. Some of the initial figures (please see specific comments below) show HFs with YFP (or lacZ) labelled SGs but without any bulge labelling. Therefore, it is not clear whether bulge cells must first divide within the bulge to then migrate towards the SG progenitor niche (as implied from figure 5), or whether bulge contribution to these upper areas can take place without the prerequisite of division within the bulge (as implied from figure 1 and supp fig 1, where HFs with YFP+ labelled IU and SG cells, but no labelled bulge cell, are shown). Both scenarios would be valid. However, clearly showing which case is predominant, or even if both cases occur in a similar proportion, would further strengthen the conclusions of their work.

Specific Comments:

- Fig 1G: The HF shown does not seem to be in telogen, morphologically it looks like an anagen HF. This should either be corrected or stated otherwise.

- Also, no YFP+ labelled cells can be seen in the bulge region (Fig 1G). Is this because recombination did not occur in the bulge, or is it because recombined bulge cells very quickly migrated to the SG region without any prior division? Do the authors observe any bulge proliferation at these same timepoints after treatment with tamoxifen? Their very nice results from Figure 5 would indicate that bulge proliferation is taking place prior to migration towards the lower tip of the SG or the UI. In this sense, shouldn't by definition any HF that contains YFP+ cells in the SG and UI have YFP+ cells in the bulge? This should be addressed and discussed.

- Likewise, a significant number of YFP+ (or lacZ+) cells is observed at 5 or 7d post recombination in the SGs and UI. The quantification results (Fig 1K) seem to support a coexistence of YFP tagged bulge cells and SG cells in a large number of HF (as expected from the results shown in Figure 5) yet the wholemount examples the authors have shown barely indicate any coexistence (i.e. the HF contain YFP cells in the SG but barely no cells in their bulge regions). Is this because of cells that have migrated into these areas from the bulge immediately started to proliferate once there? Is the turnover in the UI and SG so rapid that single labelled bulge cells migrating into the UI and SG regions would almost fill the entire areas only after 5-7 days post recombination? Basically, are these cells proliferating in the UI and SG at these same timepoints analyzed? One would expect so from the results shown, but thisshould be included (a simple staining for a proliferation marker colocalizing with YFP should be sufficient). If the authors should not see proliferation of YFP+ cells in the SG and UI at these time points, how could they explain then such high number of YFP labelled cells at these early timepoints?

- Fig S1D: as in Fig 1G again the SG are filled with Lacz+ cells but no blue cells are observed in the bulge region. Can the authors rule out 100% that there is no recombination in the UI and SGs? The wholemounts of Cre expression are not too entirely unequivocal to this respect, and one can hint, by looking carefully at figure 1B, some positive cells in the UI and even faint expression in some nuclei in the SGs. Perhaps higher magnification of these stainings should be shown in these areas (UI and SG) to completely rule out any recombination. It is true though that the authors address this issue in

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Supp. Fig 2b, but these sorting experiments cannot rule out that at day 2 recombination is only visible in the bulge but that at day 6 recombination can also occur in the isthmus regions (as for instance the amount of Cre expressed in the bulge is higher at d6 than d2, a similar thing could be happening in the UI). The authors might imply that the Cre band observed in the Isthmus at d6 comes from cells that have migrated from the bulge, but how can they know this for sure? Perhaps the conclusions of these results should be tamed.

- In figure 2B-D, H when the authors mention that ®this demonstrates that in addition to their activation for the tissue regeneration, labeled bulge cells are able to replenish the HF SC pool over longer time periods in adult skin and this process seems to involve a symmetric cell division® they should cite the work of Zhang et al (2009) in which they reach the same conclusion.

- In figure 3C it is not clear that the YFP is colocalizing with SCD1. If some of the enlarged SGs of K14deltaNlef1 mice originate from YFP labelled bulge cells, SCD1+ cells should also be YFP+. Is this the case? In figure 3C green and red fluorescence do not seem to colocalize in the same cells suggesting that the YFP+ labelled cells are barely contributing to de novo SG formation. Clearer colocalization of YFP and SCD1 should be shown.

- The wholemount immunostainign for K15 and TnC show a broader pattern of expression than expected (i.e. TnC and K15 are usually confined uniquely to the bulge area and not to the hair germ region as shown in Fig 3A and 3C). This does not change at all the conclusions that the authors make (since you can clearly see that the YFP labelled cells are within the bulge), but perhaps enhanced stainings could be shown.

1st Revision - Authors' Response 24 May 2011

Reply to Referees

Referee #1:

"In this manuscript, the authors analyze the contribution of bulge cells to multiple differentiated progeny of pilosebaceous unit. In particular, they focus on the contribution of bulge cells to the sebaceous gland. To do so, they cross a mouse line expressing a tamoxifen inducible cre recombinase under the bulge specific K15 promoter with reporter mouse lines. Lineage tracing analysis reveals that bulge cell progeny contribute to the renewal of the sebaceous gland during skin homeostasis independent of the hair cycle stage. Through careful analysis of gene expression and the time-course of bulge cell progeny out of the CD34 region of the bulge, the authors demonstrate how bulge cells progress through multiple regions of the follicle as they exit the stem cell niche. In addition, the authors provide strong evidence that expression of the dominant active ΔNLef1 transgene results in the contribution of bulge cell progeny to ectopic sebaceous gland formation. This manuscript addresses several important concepts that are important for the stem cell and skin field, including the relationship of stem cells to their progeny and Wnt signaling contributes to progenitor formation. In general, the manuscript is well written and the experimental data presented are convincing. The in vivo imaging experiments, careful lineage tracing, and various mouse models utilized by the authors provide innovative and informative data regarding the activity of stem cells in the skin. In some cases, the conclusions are not fully supported by the data presented. With additional data and textual changes, this manuscript will be appropriate for the EMBO Journal"

We thank the reviewer for his/her very positive comments. The constructive suggestions on our work helped to improve the quality of the manuscript. We have performed a variety of experiments to further strengthen our initial results. Additionally, we have revised the text to avoid speculations that are not fully supported by the data. Our specific comments to the issues raised by the reviewer are outlined below.

"Specific comments are below.

1. For both reporter lines, the authors should perform a detailed analysis of labeling after 3 days of

induction to validate the absence of reporter expression in the basal epidermis throughout the entire back skin and within the tail upon tamoxifen treatment."

A: As suggested by the reviewer we quantified the number of labelled cells 3 days following Cre activation by tamoxifen application in A_K15CreER^{low}/R26RYFP and A_K15CreER^{low}/R26RLacZ mice which are presented in Supplementary Figure S1F for tail skin. The results reveal that within an area covering 20 HFs about 1 positive cell was detected within the interfollicular epidermis (IFE) in A_K15CreER^{low}/R26RLacZ mice whereas one YFP+ cell/clone was seen in an area spanning about 220 HF in A_K15CreER^{low}/R26RYFP mice.
We also tried hard to determine marker-positive keratinocytes within the IFE of back skin whole-mounts. The localisation of YFP+ keratinocytes was restricted to the HF bulge in back skin of A_K15CreER^{low}/R26RYFP mice. The high density of HFs in back skin samples and some crystalline residues of the X-Gal dye did not allow to unequivocally determine the number of positive cells in A_K15CreER^{low}/R26RLacZ.

"1. Figure 1. a. It would be useful to know how many cells are labeled at each timepoint. Is there an increase in the number of cells labeled at each timepoint? This would indicate that the cells are proliferating during their transition to the sebaceous gland."

A: We have addressed this important question and counted the total number of labelled cells at 3, 8 and 180 days following Cre activation. As demonstrated in the new Figure 1L there is a clear increase in the number of labelled cells per pilosebaceous unit over time. This indicates that the labelled cells indeed proliferate during transition from the bulge towards the sebaceous gland.

"b. Do labeled cells contribute to the hair germ during this experiment?"

A: As demonstrated in Supplementary Figures S3 and S4 bulge derived progeny are able to contribute to all lineages of the HF following entry into a new round of the hair cycle. As expected, we do detect labelled cells within the hair germ at the beginning of a new anagen phase. A representative image for this observation is now shown in Supplementary Figure S4A.
The question if recombinant Cre is also targeted to the hair germ is difficult to address. Very few YFP+ keratinocytes were detected within the hair germ at day 3 and 5 post Cre activation at the beginning of anagen. However, given the high mobility of bulge cells at this stage of the hair cycle (see also Hsu et al, 2011; Jaks et al, 2008; Greco et al, 2009) we can not clearly distinguish if labelling occurred within the bulge or the hair germ region. Furthermore, the number of labelled cells within the hair germ was just too low and therefore prevented their further characterisation, e.g. by FACS.

"c. The authors state throughout the manuscript that cells are seen within the sebaceous gland duct. How is this visualized? Examples of this labeling are not evident from the data presented."

A: We agree with the reviewer that it can be challenging to clearly localise the ductal region of sebaceous glands in epidermal whole mounts. We tried extensively to demonstrate co-staining with established SG duct marker molecules, e.g. K6a (Gu and Coulombe, 2008). Unfortunately, the staining pattern observed in these experiments was not always unequivocal and consequentially were not included in this manuscript.
Nevertheless, we are convinced that it is important for the interpretation of the data to distinguish different regions of the pilosebaceous unit to allocate clones of labelled cells and obtain a detailed picture of where expansion of the labelled cell population occurs within the tissue. Therefore, we defined the region of the sebaceous duct precisely to the junction between sebaceous gland and junctional zone of the hair follicle and a representative image is shown in Supplementary Figure S1D. To better communicate this point within the manuscript, we have now stated in the text on page 8 how we define the region of the SG duct and have highlighted the area of the SG duct (D) within the model presented in new Figure 1A.

"d. The labeling in Figure 1 is referred to as "single" bulge cells. Because single refers to one cell, perhaps individual would be a better term."

A: We have changed the text on page 8 accordingly.

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"e. The reference to the control data in Supplemental Figure 2 should be moved to after the first paragraph in the results section before the discussion of the lineage tracing results."

A: This point could not be addressed because there was no reference to the control data in Supplementary Figure 2 found in the text.

"2. a. The authors conclude from the data within figure 2 that "labeled bulge cells are able to replenish the HF SC pool over long periods in adult skin and this process seems to involve symmetric cell division". The use of "symmetric" cell division is not clear: are you referring to the axis/direction of division or the differential cell fate of stem cells to self-renewal or differentiation? This conclusion may be based on the in vivo lineage tracing experiment in Figure 5. Further analysis of these live imaging data (quantification of the direction of cell division) or clarification by what symmetric division means is required. Furthermore, quantification of the number of cells labeled in the bulge over time (see points above for Figure 1) may make the "symmetric cell division" statement more clear."

A: We would like to apologise to the reviewer for the confusion caused. In our revised manuscript we state precisely within the text on page 11 and 12 that the term "symmetric cell division" is used for the decision of cell fate (stem cell and self-renewal vs. transient amplifying cell and lineage commitment) and does not refer to the direction of axis during cell division. We do believe though that the issue of orientation of the cell division plane within the bulge SC area is an important one that could be addressed by our ex vivo time lapse experiments in the future but is out of the scope of this manuscript. To further strengthen the important finding of proliferation of labelled bulge stem cells we have followed the advice of the reviewer and quantified the number of labelled cells/size of labelled cell clones within the HF bulge at 3, 8 and 180 days of tracing. These data demonstrate that there is an increase in the size of labelled clones over time with detection of largest clones at 180 days post Cre activation. These results are presented in new Figure 2H and clearly show that there is indeed replenishment of labelled epidermal SCs within the HF bulge.

"b. The authors conclude that their data "argues against the possibility that genetically labeled progenitor cells are residing within and regenerate the SG. Instead the results prove that HF SCs are an important source for constant renewal of SGs". However, this conclusion is too strong based on the data presented. Since SG progenitor cells that are replenished by bulge cells would be genetically labeled with YFP or lacZ marker, the contribution of bulge cells vs progenitor cells in a long-term labeling experiment cannot distinguish between bulge cell or progenitor cell contribution. Furthermore, the data in Figure 5D demonstrate that a previously identified marker of SG progenitor cells (Blimp1) is upregulated in labeled cells after 6 days."

A: We agree with the reviewer and have now toned down our interpretation (page 9 and 10). Based on our results we can not exclude the possibility that labelled bulge progeny give rise to long-living SG progenitors that are localized close to or within the SG and are also able to renew the gland tissue. However, if this would be the main scenario one would expect an increase of labelled SGs in the long-term experiment since bulge cells contributed to the establishment of progenitors over a long period of time. This is clearly not the case as illustrated in our statistical analysis in Figure 2K, where the pattern of labelled cells is similar on day 180 post Cre activation when compared to day 8 following Cre activation (Figure 1M). Furthermore, only one of the two prominent SGs was repopulated by labelled bulge progeny in the majority of cases and no expansion of cell clones within the upper isthmus or junctional zone (JZ) was observed over time (Supplementary Figure S5E). This result would not be expected if bulge progeny would only give rise to progenitors localised close to the SG. Although our results suggest a continuous contribution of bulge progeny for SG renewal we do consider the possibility that labelled bulge cells could generate SG progenitor cells and we have therefore revised the text accordingly.

"3. Figure 3. a. The data in Figure 3 could be improved with the use of a bulge cell marker. Given the abnormal appearance of the follicles in K14ΔNLef1 mice, the location of the bulge is not evident."

A: We have performed an extensive analysis of various bulge markers on epidermal whole-mounts of K14ΔNLef1 mice. These immunostainings reveal that marker expression is different from

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wildtype skin samples and not specifically confined to the bulge region in epidermis of K14ΔNLef1 mice. We now provide representative images for localisation of CD34 and α6 integrin within Figure 3A,B. In addition, immunofluorescent staining with an antibody against keratin 15 is shown in Figure 6A, demonstrating localisation of keratin 15 to some bulge cells but also outside the deformed bulge region.

"b. Quantification of these data similar to that performed in Figure 1 in WT mice is needed. Additionally, the number of cells in each region of the follicle (bulge, hair germ vs sebaceous gland) should be quantified for each timepoint."

A: We agree with the reviewer that it would be very interesting to obtain more detailed information on the precise localisation of bulge progeny at different time points of tracing in K14ΔNLef1 mutant mice. However, the quantification of labelled cells within the different compartments could not be performed in K14ΔNLef1 mice as done with wildtype animals due to the following reasons: First, the different cellular compartments are not confined anymore as documented by abnormal expression of marker molecules, e.g. bulge marker and α6 integrin (Figure 3A, B and Figure 6A). No hair germ is observed within the deformed HFs of K14ΔNLef1 mice (see also Figure 3). Secondly, a dramatic increase in proliferation is detected in all compartments along the abnormal HFs structures as shown in Figure 3C (see also Niemann et al, 2002 and Braun et al, 2003). Finally, the abnormal HF structures do highly vary in shape, size and morphology and are not as uniform as HF in wildtype skin. Therefore, analysis of number and localization of labelled cells is hampered within these complex structures.
However, to address this issue of exact localisation of labelled epidermal cells within K14ΔNLef1 mice, FACS experiments were performed to determine if the number of YFP+ keratinocytes localised to the bulge (CD34) and the MTS24 compartment changes during tracing. As demonstrated in new Supplementary Figure S6E-K there is now significant change of YFP+ cell pool within the CD34+ bulge cells. In contrast, an increase in labelled cells was detected within the cell compartment that was immunolabelled with MTS24. This result provides further proof that labelled bulge progeny contribute to the formation of ectopic SG and new progenitor compartments in K14ΔNLef1 mice. As expected, an overall increase within the number of labelled keratinocytes was also monitored in K14ΔNLef1 mice when compared to wildtype control mice (Supplementary Figure S6K).

"4. Figure 4. Quantification of the number of cells in each cell location (CD34/LRC/K15, MTS24, Lrig1 vs SCD1) at each timepoint should be performed."

A: Based on the suggestion of the reviewer we now have quantified the number of labelled cells within the bulge, the upper isthmus, the junctional zone (JZ) of the HF and the SG duct and within the SG. In addition, we have subdivided the labelled cells within the SG according to their localisation at the lower tip (LTip), top region of the SG (Top), at the inner periphery (P) and the central part (C) of the gland. We included these quantifications within new Figures 2H,I and Figure 5J (for Bulge, SG and distribution within the SG) and Supplementary Figure S5E,F (JZ and SG duct). As expected, these results demonstrate an increase in the number of cells within the regions of the bulge, SG duct and SG in whole-mounts analysed at 8 days following Cre activation when compared to samples 3 days following Cre activation. Together with a detailed temporal and spatial analysis of the process of SG renewal by bulge SCs and the data of the time lapse ex vivo studies, these results strongly suggest that the SG is repopulated by labelled HF bulge cells at the inner periphery of the gland prior the expansion of labelled cell clones at the lower tip of the gland. We now include a model summarising our main observations in Figure 5K.

"5. Figure 5. a. The data in Figure 5A may be more appropriate within Figure 1 or Figure 2. Additionally, the quantification of these data in Figure S5a should be included within the main manuscript."

A: Based on the suggestion of the reviewer and the recommendation made by Reviewer #2 we highlighted the ex vivo tracing experiments and the analysis of proliferation and migration of bulge SC is now presented as an own figure and as a separate chapter within the results. As suggested by the reviewer we included the quantification of the time lapse analysis of individual bulge SCs within the main figures (Figure 5B). However, it appears to us that the documentation of the renewal of the SG by bulge SC progeny and the transition through the progenitor compartments within the

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pilosebaceous unit requires its presentation prior to the characterisation of mechanistic details (proliferation and migration of bulge SCs) as shown in Figure 5.

"b. The real time PCR data in Figure 5C and 5D is not clear. Do the labels on the X axis indicate sorted cell populations? If so, the use of multiple markers should be used (alpha 6 integrin and CD34; MTS24 and alpha 6 integrin). The fold increase D5/D2 would be more informative regarding how gene expression changes in YFP+ cells over time."

A: We would like to apologise for the confusion caused and have now improved the legend of the Figures (now Figure 4L,M) and the text on page 15 to better communicate the design of these particular experiments. The label on the x-axis does indeed indicate sorted cell populations and, as suggested by the reviewer, CD34 and YFP populations were sorted in conjunction with alpha 6 integrin. Experimental details did not allow sorting with MTS24 in conjunction with antibodies directed against alpha 6 integrin. This sort reflects the total MTS24+ cell population since MTS24 antigen/Plet1 is also expressed by alpha 6 integrin low or negative cells (Raymond et al, 2010). To demonstrate sorting efficiency, marker expression was determined by qRT-PCR and these results are now presented in Supplementary Figure S4K,L.
The analysis of gene expression within the α6 integrin+/YFP+ cell population was done by qRT-PCR following sorting of the cells. The results of these experiments are illustrated in Figure 4M where changes in the lineage marker Lrig1, Lgr6 and Blimp1 were investigated in detail.

"6. Figure 6. The source of material for analysis of genes expressed by bulge cells or non-bulge cells is not clear. Sorted alpha6 integrin+, YFP+ cells should be analyzed in this experiment."

A: In this particular experiment, RNA of total skin of K14ΔNLef1 mice was analysed for marker expression at different time points of development in comparison to wildtype skin samples. We have now clearly communicated the source of material within the text on page 17. In addition, we now include experiments analysing CD34 (bulge) and MTS24/Plet1 (upper isthmus) marker within YFP+ cell population by FACS. These data are shown in Supplementary Figure S6E-K and reveal: a) an overall increase in number of YFP+ cells in K14ΔNLef1 mice when compared to wildtype littermate control animals (Supplementary Figure S6K); b) an increase in YFP+ cells within the MTS24/Plet1 compartment at day 5 when compared to day 2 post Cre activation in K14ΔNLef1 mice (Supplementary Figure S6H,J); c) no overall changes of YFP+ cells within the CD34+ cell pool at different tracing time points in K14ΔNLef1 mice (Supplementary Figure S6G,I).

"7. Figure 7. The K15ΔNLef1 transgenic mouse model provides strong evidence that the bulge cells contribute to ectopic SG formation. The CD34+ cells near the sebaceous gland are not clear in Figure 7F. Furthermore, the significance of CD34+ cells near the gland is not clear either. Are other bulge SC markers expressed in this location?"

A: We are glad that the reviewer finds that the analysis of the K15ΔNLef1 mice strengthens our data on the important role of bulge SC for constant SG renewal. We have now improved the quality of the image in Figure 7 to clearly show localisation of CD34 to the duct region of abnormal SGs. In addition, we also identified mislocalisation of keratin 15 and tenascin C above the bulge region. We now included a new image demonstrating expression of keratin 15 at sites of abnormal and de novo SGs in Figure 7H. We have also revised the text on page 18 to better communicate and elucidate this interesting observation suggesting a more complex function of mutant Lef1 for the regulation of HF morphology.

"8. Abstract. The data presented do not support the conclusion within the abstract that "this process of de novo SG formation involves the establishment of new progenitor niches". Furthermore, how these data show "the recapitulation of early steps of tissue morphogenesis" is not clear."

A: We have changed the abstract to state that "this process of de novo SG formation is accompanied by the establishment of new progenitor niches" as revealed by the expression pattern of Lrig1 and MTS24/Plet1 in K14ΔNLef1 transgenic mice.
We are convinced that the process of ectopic SG formation reapplies the same basic sequence of molecular and cellular events as seen during SG morphogenesis. This is supported by observations made by other groups, where a recapitulation of the HF differentiation program was also seen in

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mouse models investigating development of ectopic HFs (e.g. Lo Celso et al, 2004; Silva-Vargas et al, 2005; Youssef et al, 2010).
Since the molecular signals underlying the process of SG morphogenesis are not known yet, we have studied the molecular signature coupled to the formation of ectopic SG formation in K14ΔNLef1 mice. We changed the text on page 18 to better communicate our observations and interpretation of our data.
We agree that in the future, it will be important to develop new genetic and molecular tools that allow to study the process of SG morphogenesis in much more detail and to identify essential signals required for SG formation. Furthermore, it will be interesting to determine the role of these signals for patho-physiological conditions, e.g. sebaceous tumours or acne.

Minor points:

"1. The SD abbreviation is confusing. SG duct would be less confusing."

A: We have followed the advice of the reviewer and have changed the abbreviation SD to SG duct (and used "D" as abbreviation within the model in Figure 1A) within our revised manuscript.

"2. The verb usage for progeny is singular and should be plural. (see page 8, first paragraph)."

A: We apologise for this mistake and have changed the text accordingly.

"3. On page 8, first paragraph, the following sentence should be edited: "frequency of labeling of bulge cells was lower in the back when compared to tail epidermis" to "frequency of labeling of bulge cells was lower in the follicles within the backskin when compared to those in the tail epidermis"."

A: We changed this sentence on page 8 as suggested by the reviewer.

"4. The text used to label axes of the graphs in the Figures should be increased."

A: As suggested by the reviewer we now have improved the labelling within the different graphs.

"5. Fig S1 is missing subheadings (A, B, C...)"

A: We apologise for any confusion caused and have now included subheadings and correct labelling of the panels in Supplementary Figure S1A-F.

Referee #2:

"This study sets out to investigate cell fate and lineage in the hair follicle and sebaceous gland (SG) in transgenic mouse epidermis and how this is altered by an activated wnt pathway mutant.
The introduction highlights the two current models of SG homeostasis, ie that they are maintained by a dedicated SG progenitor population or by the proliferation of stem cells in the hair follicle bulge.
The complexity of hair follicle stem cell markers can be bewildering to a non specialist and it would be worth including a diagram of the follicle to summarize the different cell populations and their location."

A: We would like to thank the reviewer for this suggestion. We now have included a scheme of the pilosebaceous unit summarising important stem and progenitor cell populations and different regions within the pilosebaceous unit in Figure 1A.

"The study begins with the generation of new transgenic lines using the keratin15 promoter to drive the expression of an inducible cre. One line labels most of the follicle bulge whilst a low expressing line permits cell labeling at clonal density, providing a powerful tool for the authors to investigate bulge cell fate. Reporter expression is analyzed in wholemount which is a real strength, and both

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bgal and YFP reporters are shown to give consistent results. It would be helpful to state how many hair follicles were scored for YFP with the uninduced K15 low strain animals, as a lack of background recombination in SG is crucial for interpretation of these experiments."

A: We are glad that the reviewer appreciates the complex experimental design analysing epidermal whole-mounts and that he points out that application of two different reporter lines giving consistent results is a real strength of our manuscript. Based on the suggestion made by the reviewer we now have scored YFP positive cells in uninduced A_K15CreER^{low} mice. These quantifications clearly show that there is no recombination in uninduced A_K15CreER^{low}/R26RYFP mice and a negligible background recombination within the SGs of A_K15CreER^{low}/R26RLacZ mice (day 3: 1 out of 774 HFs and day 8: 1 out of 1107 HFs). These important results clearly show the specificity of bulge SC labelling following tamoxifen administration and are now presented as part of Supplementary Figure S2J,K.

"The fate of cells following low level induction is then tracked. 3 days after induction, 90% of the labeled cells are located within the bulge, with the remainder in the sebaceous gland or "complex", ie in the bulge, sebaceous gland and sebaceous duct. The proportion of complex hair follicles rises to 40% at 8 days. This data is interesting but would be strengthened by counting the size of the groups of labeled cells, which should represent clones if single cells are present at the start of the experiment. Crucially are multicellular clones which extend from the bulge to the SG seen at day 8? This may be the case in Fig 1I, for example, but it would help to show appropriate views from 3D reconstructed Z stacks to clarify this issue. In the current presentation the data do not "clearly indicate that the HF bulge contains a population of multipotent SCs contributing to the renewal of the SG during skin homeostasis.""

A: We agree with the reviewer that the issue of analysing the size of labelled clones is an important one. Therefore, we have determined the number of keratinocytes in labelled cell clones at different locations within the pilosebaceous units at day 3, day 8 and day 180 following Cre activation. The results convincingly show an increase in clone size within the bulge, the SG duct and within the SG when comparing day 8 of tracing with day 3 post Cre activation. These new data are now presented in Figure 2H (Bulge), Figure 2I (SG), Supplementary Figure S5E (JZ) and Supplementary Figure S5F (SG duct).
Additionally, we have also grouped labelled cells within the SG according to their localisation at the lower tip (Ltip), on the top of the gland (Top), at the inner periphery (P) and the central part (C) of the gland (see also scheme in Figure 5K). As shown in Figure 5J, an increase in number of positive cells/clone size is detected within these different cell populations localised within the SG at later tracing time points. Interestingly, at the lower tip of the gland clonal expansion is evident at day 8 following Cre activation and the size of clones are larger when compared to clones at the periphery, central part or anterior region of the SG. Together with a detailed temporal and spatial analysis of the process of SG renewal by bulge SCs and the data from the ex vivo tracing experiments, these results suggest that the SG is repopulated by labelled HF bulge cells at the inner periphery of the gland prior the expansion of the labelled cell clones at the lower tip of the gland. We now include a model summarising our main observations in Figure 5K. As expected, there is an increase in number of labelled cells in each location analysed in long-term tracing experiments (day 180) when compared to day 8 post Cre activation. The statistical data of the experiments are now shown in Figure 5J und discussed in the main text on page 16 and 22 (results and discussion).

Furthermore, based on the recommendation made by the reviewer, we have performed 3D rendering of the Z stack projections analysing labelled cell clones contributing to SG and HF renewal. As already implied by our initial results, the majority of labelled HF does not contain continuous cell clones spanning a region from bulge towards the SG as seen in the process of HF renewal during anagen (new Supplementary Figure S4E-G). Instead, a few individual cells can be monitored that are lined up from the bulge towards the SG duct. As also demonstrated in our *ex vivo* live cell imaging experiments (Figure 5A-B), there is fast migration of bulge progeny towards the junctional zone of the HF upon cell division of bulge SCs. In contrast to the process of HF renewal, where constantly new cells need to be generated to establish a new hair, no expansion of the cell pool is required for SG renewal unless bulge progeny reach the area of the gland. Indeed, propagation of labelled cells is seen within the SG. This observation is also strengthened by the calculation of the size of labelled clones which is largest within the SG (new Figure 2I, new Supplementary Figure

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S4J, new Supplementary Figure S5E,F). We have now included images of 3D reconstructions in new Figure 2A-G to document these important findings.
Furthermore, we have revised the text to better communicate the important role of migration of bulge progeny.

"The characterisation of the cells targeted by the K15 (suppl fig 2) might be better moved before figure 1."

A: We would like to thank the reviewer for his/her suggestion. However, we believe that the introduction of the different mouse models and the important novel observations should be presented before further characterisation of labelled cells and presentation of details on the cellular mechanism of SG renewal by bulge cells. Furthermore, changing the order of presentation would require introducing the different progenitor compartment before actually linking the observation of labelled bulge progeny to these regions. Therefore, we respectfully disagree with the reviewer. It is our opinion, that the manuscript would not improve by moving the figures as suggested.

"The discussion about clones in different phases of the hair cycle and the presence of bulge derived cells (Suppl Fig 3) would be strengthened by 3D views showing contiguous clones extending from the bulge at a sufficient magnification to be clear to the reader."

A: As suggested by the reviewer we performed 3D reconstruction of labelled whole-mounts from different stages of the hair cycle. As stated above, in the majority of cases there are no contiguous clones expanding from the bulge towards the SG due to the fast migration of bulge progeny. The high cellular turn over within the SG requires propagation of bulge derived progeny within the SG but not within the isthmus region. Therefore, labelled bulge cells migrate swiftly upwards to expand once they reached the SG duct and SG. This is strengthened by the data presented in new Figure 5C-I where many more proliferative cells were detected at the periphery of the SG when compared to the bulge and isthmus region. Also, it is not known if bulge SC are also involved in renewal of the isthmus compartment at all. Recently, other progenitor and stem cells have been localised to this compartment and therefore, could be involved in replenishment of isthmus cells (Nijhof et al., 2006; Snippert et al., 2010).
Interestingly, as demonstrated in new Supplementary Figure S4J an increase in mobilisation of bulge progeny and elevated number of labelled cells within the SG were observed when labelling of bulge SCs was done during telogen to anagen transition. At this particular time point it was possible to detect a trail of labelled cells expanding from the bulge towards the SG. As expected, a trail of labelled cells was also seen from the bulge downward to generate the new hair. We have included images presenting the 3D reconstructed Z stacks for tracing during anagen in new Supplementary Figure S4E-G.

"In the long term labeling studies, Fig2, there is an interesting increase in the number of cells/bulge. If continual replacement of SG cells by bulge progeny is occurring, there should be clones extending from the bulge into each labeled SG at 180 days. It is not clear from the images presented if this is the case. How does the size of the bulge derived clones compare with that at 3 and 8 days?
The presence of clones with no connection to the bulge at 180 days argues that the SG contains a self maintaining population of progenitors. This is an important observation that should be discussed. Again it would be helpful to show images of sufficient quality to convince the reader that these clones have no connection with their size and compare the size distribution of these clones with that at early time points."

A: The analysis of the size of different clones expanding from the bulge is indeed important. We have determined the number of cells/clone size at different locations within the pilosebaceous unit at different time points following Cre activation (3, 8 and 180 days). The new results are presented within the revised version of our manuscript in Figure 2H,I, Supplementary Figure S5E,F and within the result chapter on page 12. Interestingly, the pattern of distribution of labelled cells (especially SG and SG duct pattern) is very similar between 8 and 180 days post Cre activation (Figure 1M and 2K). Importantly, no increase in the number of labelled cells was seen in the JZ at 180 days following tamoxifen administration when compared to 8 days of tracing time (Supplementary Figure S5E). These data exclude the possibility of propagation and of labelling SG precursor cells at the

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isthmus/junctional zone. In contrast, there is an increase in clone size observed within the bulge and the SG (Figure 1H,I) at 180 days post Cre activation.
As stated above, there are no contiguous cell clones expanding from the HF bulge towards the SG and this is due to fast migration of labelled cells as convincingly shown in the ex vivo tracing experiments (Figure 5A,B). These data were even further strengthened by demonstrating higher proliferation rate of keratinocytes at the SG duct region and the periphery of the SG when compared to bulge and upper isthmus as illustrated now in Figure 5C-I.

"The authors then move on to investigate the lineage tracing in mice expressing a dominant negative Lef1 transcription factor. Bulge derived cells contribute to the ectopic SGs induced by expression of the mutant (fig 3). It would be interesting to know if labelled SG clones with no connection with the bulge persist at the 90 day time point."

A: Although labelled keratinocytes were not analysed as extensively at 90 days post Cre activation in K14ΔNLef1 mice, the majority of deformed HF structures show labelled cell clones expanding from the abnormal bulge region.

"The expression of markers in labelled cells is then investigated. The low power of the images in Fig 4 makes it difficult for the reader to confirm that individual labelled cells do indeed express the markers shown, some higher power insets which avoid oversaturation of the green channel would help here. Why is the FACS data included in 5B-D not presented as part of Fig 4?"

A: Based on the helpful comments made be the reviewer we have now included higher magnifications of images presenting YFP positive cells co-stained with marker molecules Lrig1 and SCD1. These data are shown in Supplementary Figure S5A,C. We have also followed the advice of the reviewer and present the FACS data as part of Figure 4 (Figure 4L,M) in the revised version of the manuscript.

"Fig 5 A and the associated supplementary Fig 5 are a highly impressive experiment using time lapse imaging to track cell fate in explants. This should be highlighted in the main text and deserves a main figure on its own. The data should be presented in more detail and discussed more fully. In the current version the impact of the live imaging is lost and it is unclear why it is placed in the middle of a discussion about markers. Minor point: The same paper is Lu 2006 in the text and Lu 2007 in the reference list."

A: We are glad that the reviewer appreciates the time lapse imaging of whole-mount explants as innovative and impressive experiments. We followed the advice and show these experiments now in Figure 5 in more detail. Therefore, we moved the statistics from the Supplementary Figure into Figure 5 and we present the ex vivo lineage tracing data within an own chapter within the results ("Proliferation and migration of bulge SCs").
We apologise for the mistake and have changed the reference within the text of the manuscript on page 15 to "Lu et al, 2007".

"Fig 6 is a molecular characterisation of cells in the mutant phenotype. Given the complexity of the system the interpretation of the significance of the multiple markers examined is more speculative (the mutant may alter the expression of these genes independently of changes in cell behaviour). It is unclear what this level of analysis of the "neomorphic" mutant phenotype adds to what is already a strong story about cell fate in normal homeostasis. Similar comments apply to Fig7, which shows the effect of expressing the mutant from a K15 promoter."

A: We are convinced that the molecular characterisation of the development of new SGs seen in two transgenic mouse models is an important issue. The K14ΔNLef1 mouse model allows investigating the process of de novo formation of SGs in much detail. Until now, hardly anything was known about the molecular mechanisms underlying SG morphogenesis. Therefore, analysing the expression of various marker molecules during formation of ectopic SGs provides novel information on a potential molecular signature underlying the process of SG morphogenesis. For instance, the exciting observation of the establishment of new SG progenitor compartments adjacent to the newly formed SGs, which was also reflected in the qRT-PCR analysis, points to a more general mechanism and could be of great importance for the field of regenerative medicine.

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"In the discussion citing the Langton 2008 paper seems inappropriate, as this shows that epidermis heals without input from hair follicles in Edar pathway mutant mice."

A: We have eliminated this citation in the discussion of our manuscript.

"Overall there is some very interesting data here. The authors could do a better job of presenting this however, and given the limited space available in a single paper, might perhaps focus more on normal homeostasis than the "artefactual" generation of SG by transgenic expression of a mutant protein. The paper hinges around 3D imaging of clones, and would be strengthened by 3D rendering of Z stacks to illustrate the key points about the clones: the current images are difficult to interpret and are often at too low a power to assess the two color immunofluorescence.
The standard of English is poor, with multiple spelling and grammatical errors throughout. I would urge the authors to revise the text with the help of someone fluent in English. If the issues discusses are addressed I would support publication in the EMBO Journal."

A: We thank the reviewer that he/she is positive about the manuscript. In our revised version of the manuscript we have focused on the characterisation of size and distribution of labelled cell clones and performed 3D reconstructions of Z-stacks of confocal images. The overall quality of images was improved and the process of SG renewal by bulge SCs was investigated in more detail. We have addressed all the issues raised by the reviewer and hope that the manuscript is now suitable for publication in the EMBO Journal.

Referee #3:

"In this work, Peterson et al have addressed the contribution of hair follicle stem cells to the different compartments of the pilosebaceous units, and show that bulge cells contribute to the turnover of SGs and the stem cell niches within the isthmus region. The specific contribution of bulge cells to the different compartments of the hair follicle, and their dynamic behavior, is still under in the field. Previous works have suggested that the bulge contributes to the turn-over of the SGs, however, how this occurs, and whether this is dependent on a specific phase of the HF had not been studied in detail.
The authors here provide compelling and elegant evidence for the complex dynamic behavior of the bulge stem cells, showing how and when they contribute to the SG and to the isthmus regions. Their in vivo time-lapse imaging is simply superb, and to my knowledge will be the clearest and most unequivocal evidence provided so far in the field for a direct contribution (i.e. migration) of bulge stem cells to the SG and UI. In addition, in it is still under debate whether bulge stem cells divide symmetrically or asymmetrically. Whereas this might be clearer for the contribution of bulge cells to the HF growth per se, the authors now convincingly show that the bulge cells proliferate symmetrically to subsequently migrate towards the SG progenitor niches for contributing to SG renewal."

A: We thank the reviewer for his/her positive comments and his/her acknowledgement of the exciting and novel results presented within the manuscript.

"I only have one general criticism to the manuscript. Some of the initial figures (please see specific comments below) show HFs with YFP (or lacZ) labelled SGs but without any bulge labelling. Therefore, it is not clear whether bulge cells must first divide within the bulge to then migrate towards the SG progenitor niche (as implied from figure 5), or whether bulge contribution to these upper areas can take place without the prerequisite of division within the bulge (as implied from figure 1 and supp fig 1, where HFs with YFP+ labelled IU and SG cells, but no labelled bulge cell, are shown). Both scenarios would be valid. However, clearly showing which case is predominant, or even if both cases occur in a similar proportion, would further strengthen the conclusions of their work."

A: We thank the reviewer for this helpful comment. We have analysed the distribution of labelled cells within the pilosebaceous unit at different time points following Cre activation. As

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demonstrated in Figure 1M, about 8% and 14% of pilosebaceous units exhibit labelled cells within the SG and SG duct only at 3 days and 8 days of tracing, respectively. Clearly, this group is outnumbered by the bulge only and complex (bulge+SG+SG duct) pattern of distribution. It has been shown by various groups that the HF bulge constitutes a rather heterogeneous stem cell compartment accommodating quiescent as well as more active SCs. Therefore, it is possible that more mobile bulge SCs are labelled in our transgenic approach and therefore, migrating cells are targeted that did not divide within the bulge prior migration. Here, the CreER system does not allow to determine if the SG+SG duct only pattern results from labelled bulge SCs that have migrated towards the gland without prior cell division or labelling SG cells locally.
The majority of labelled bulge SCs do divide before progeny is migrating upwards toward the isthmus and junctional zone. This is reflected on one hand by the increase of the number of labelled cells within the bulge over time (Figure 2H) and on the other hand on the increase of labelled cells within the complex pattern during tracing time (Figure 1M). To clearly communicate and demonstrate these results more convincingly we have applied changes to Figure 1I and the text.

Specific Comments:

"- Fig 1G: The HF shown does not seem to be in telogen, morphologically it looks like an anagen HF. This should either be corrected or stated otherwise."

A: Based on the reviewer's comment we carefully examined the original image. This HF is indeed a telogen follicle (new Figure 1H).

"- Also, no YFP+ labelled cells can be seen in the bulge region (Fig 1G). Is this because recombination did not occur in the bulge, or is it because recombined bulge cells very quickly migrated to the SG region without any prior division? Do the authors observe any bulge proliferation at these same timepoints after treatment with tamoxifen? Their very nice results from Figure 5 would indicate that bulge proliferation is taking place prior to migration towards the lower tip of the SG or the UI. In this sense, shouldn't by definition any HF that contains YFP+ cells in the SG and UI have YFP+ cells in the bulge? This should be addressed and discussed."

A: We agree with the reviewer that the issue of proliferation within the bulge is indeed an important one. Based on the helpful comments made by the reviewer we now analysed proliferation within the bulge compartment in more detail. We demonstrate in new Figure 5C-E that bulge cells of telogen HFs do indeed divide. To this end we illustrate in the revised version of the manuscript telogen HFs with proliferating bulge cells and YFP labelled keratinocytes.
We do not agree with the reviewer that by definition any HF that contains labelled cells within the SG should also possess labelled cells within the bulge. In particular, given the heterogeneity of the bulge SC compartment, more mobile bulge cells could be labelled by our approach and therefore, these cells do not divide prior migration towards the SG. However, as discussed above, our data do not allow distinguishing between the two scenarios. To better communicate these data within the text we now discuss these observations in our manuscript.

"- Likewise, a significant number of YFP+ (or lacZ+) cells is observed at 5 or 7d post recombination in the SGs and UI. The quantification results (Fig 1K) seem to support a coexistence of YFP tagged bulge cells and SG cells in a large number of HF (as expected from the results shown in Figure 5) yet the wholemount examples the authors have shown barely indicate any coexistence (i.e. the HF contain YFP cells in the SG but barely no cells in their bulge regions). Is this because of cells that have migrated into these areas from the bulge immediately started to proliferate once there? Is the turnover in the UI and SG so rapid that single labelled bulge cells migrating into the UI and SG regions would almost fill the entire areas only after 5-7 days post recombination? Basically, are these cells proliferating in the UI and SG at these same timepoints analyzed? One would expect so from the results shown, but this should be included (a simple staining for a proliferation marker colocalizing with YFP should be sufficient). If the authors should not see proliferation of YFP+ cells in the SG and UI at these time points, how could they explain then such high number of YFP labelled cells at these early timepoints?"

A: We apologise for any confusion caused and have now performed additional experiments to investigate the issue of proliferation in different regions of the pilosebaceous unit in more detail (see

also above). As elaborated on the distribution of labelled cells within the pilosebaceous unit at different time points of tracing (see Figure 1M) the majority of HF contains bulge only or the complex pattern of distribution. In our revised manuscript, we have now included new images that better represent these data (Figure 1 and Figure 4).
In addition, as suggested by the reviewer we now analysed proliferation of YFP+ cells within the UI and JZ in more detail. Proliferation of YFP+ cells was seen within the UI and the JZ as shown in Figure 5F-H. As expected, cell division of labelled keratinocytes was also evident at the lower tip of the SG (Figure 5I). Furthermore, statistical analysis of the size of labelled clones did also reveal an increase in the number of positive cells at 8 and 180 days post Cre activation when compared to early time points of tracing.
As proposed by the reviewer, SG renewal is characterised by a fast cellular turn over and many proliferating cells are detected at the periphery of the glands when compared to other regions of the pilosebaceous unit. Therefore, we consider it likely that labelled bulge cells do migrate towards the gland without cell division. Alternatively, the small percentage of labelled cells detected within the SG and SG duct could be a result of labelling individual cells localised close to or within the SG. However, no expansion of labelled cell clones were observed in the UI/JZ and no increase in the percentage of labelled SG was seen in long-term experiments indicating that only a negligible number of local SG precursors might have be targeted by the transgenic approach.

"- Fig S1D: as in Fig 1G again the SG are filled with Lacz+ cells but no blue cells are observed in the bulge region. Can the authors rule out 100% that there is no recombination in the UI and SGs? The wholemounts of Cre expression are not too entirely unequivocal to this respect, and one can hint, by looking carefully at figure 1B, some positive cells in the UI and even faint expression in some nuclei in the SGs. Perhaps higher magnification of these stainings should be shown in these areas (UI and SG) to completely rule out any recombination. It is true though that the authors address this issue in Supp. Fig 2b, but these sorting experiments cannot rule out that at day 2 recombination is only visible in the bulge but that at day 6 recombination can also occur in the isthmus regions (as for instance the amount of Cre expressed in the bulge is higher at d6 than d2, a similar thing could be happening in the UI). The authors might imply that the Cre band observed in the Isthmus at d6 comes from cells that have migrated from the bulge, but how can they know this for sure? Perhaps the conclusions of these results should be tamed."

The Cre expression shown in Supplementary Figure 1B is for the higher expressing C_K15CreER^{high} line. This line was not used for the tracing experiments. We apologise for the misunderstanding caused. We now provide Cre immunostaining for the A_ K15CreER^{low} transgenic line in Supplementary Figure 1C. In this line, nuclear Cre detection was restricted to the HF bulge.
We agree with the reviewer that based on the results presented in Supplementary Figure S2I (was S2B in previous version), we can not absolutely role out the possibility of Cre recombination also occurs within the isthmus region at later time points. Therefore, we now have toned down our interpretation of these results within the chapter on page 10. However, our statistical analysis revealed that only a minority of pilosebaceous units possess SG and SG duct pattern. Furthermore, no cellular expansion of clones within the isthmus and junctional zone was detected as demonstrated in new Supplementary Figure S5C,D.

"- In figure 2B-D, H when the authors mention that ¨this demonstrates that in addition to their activation for the tissue regeneration, labeled bulge cells are able to replenish the HF SC pool over longer time periods in adult skin and this process seems to involve a symmetric cell division¨ they should cite the work of Zhang et al (2009) in which they reach the same conclusion."

A: We would like to thank the reviewer for his/her helpful suggestion. We have now included this citation on page 11.

"- In figure 3C it is not clear that the YFP is colocalizing with SCD1. If some of the enlarged SGs of K14deltaNlef1 mice originate from YFP labelled bulge cells, SCD1+ cells should also be YFP+. Is this the case? In figure 3C green and red fluorescence do not seem to colocalize in the same cells suggesting that the YFP+ labelled cells are barely contributing to de novo SG formation. Clearer colocalization of YFP and SCD1 should be shown."

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A: We agree with the reviewer and have now improved the quality of the images to clearly demonstrate co-localisation of YFP with the sebocyte marker SCD1 in Supplementary Figures S6L (for K14ΔNLef1 mice) and Supplementary Figure S5C (for wildtype mice).

"- The wholemount immunostainign for K15 and TnC show a broader pattern of expression than expected (i.e. TnC and K15 are usually confined uniquely to the bulge area and not to the hair germ region as shown in Fig 3A and 3C). This does not change at all the conclusions that the authors make (since you can clearly see that the YFP labelled cells are within the bulge), but perhaps enhanced stainings could be shown."

A: Based on this helpful comment made by the reviewer we now have improved the immunofluorescent detection of the bulge marker. We present a better image for keratin 15 in Figure 4A. Immunostainings for Tenascin C were repeated several times and the protein was not confined to the bulge region. Instead, a broader expression pattern including the hair germ was detected.

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