

# The anti-apoptotic Bcl-2 protein regulates hair follicle stem cell function

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Dear Dr. Niemann,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript and the referee reports from The EMBO Journal (attached below). The referees acknowledge that the findings are of interest. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Thus, we will not require addressing points regarding more mechanistic details experimentally. However, it will be necessary that in a revised manuscript you address all points questioning the main conclusions of the study, and all technical concerns, or points regarding the experimental design, model systems used, or data presentation.

In this case, in particular referees #2 and #4 doubt some of the major conclusions of the paper and do not think that these are fully supported by the data, in particular they seem not convinced that Bcl-2 has the reported specific roles in sbBSCs. Thus, their major points need to be addressed experimentally.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: <http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms>

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843

(<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843>)

- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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8) Regarding data quantification and statistics, please specify, where applicable, the number "n" for how many independent experiments (biological or technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: <http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis>

9) Please add up to 5 key words to the title page.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

Referee #1:

This manuscript by Geueke et al. describes the novel finding that Bcl-2 is specifically expressed in a subpopulation of hair follicle bulge stem cells, and plays an important role in the regulation of hair follicle cycling. It was previously known that Bcl-2 promotes cell survival by blocking apoptosis, is important for the survival of skin melanocytes, and contributes to the development of some cancers, including B cell lymphomas. This paper provides new insight into the importance of Bcl-2 for the maintenance of suprabasal bulge stem cells (sbBSC) in hair follicles, which are important for regulating the hair regrowth cycle. Additionally, the authors demonstrate the consequences of aberrant activation of Bcl-2 in the epidermis: complete inhibition of caspase 3-mediated apoptosis, impair hair regrowth, and aggravated development of epidermal tumors.

Overall, the authors carefully state the major conclusions from their data. Below, I suggest a few additional experiments to strengthen their proposed mechanism that Bcl-2 protects sbBSC from anoikis, which I think is currently their weakest claim. I also suggest changes to the text to make the manuscript clearer and stronger.

#### MAJOR CONCERNS

1. Clarity of experimental timing: Overall, this manuscript would benefit from more consistent and explicit description of the timing of each experiment. The authors probe several different timepoints during development and hair cycling, but do not consistently provide clear justification for their experimental design. Providing this detail will help readers better understand the precise scientific question the authors are addressing with each experiment, and the significance of the results.
2. Fig. 2d (Experiment): While the concept of this in vitro anoikis assay is logical, critical controls and/or references are missing. The data presented show that aCas3<sup>+</sup> staining increases when cultures are treated with an integrin  $\alpha 6$ -blocking antibody compared to untreated controls. However, it is not shown (or referenced) that this antibody induces keratinocyte detachment. Additionally, without an Ig control, it cannot be discerned whether this effect is caused by blocking integrin  $\alpha 6$ , or merely by the presence of antibody.
3. Fig. 2 (Experiment): The authors claim in the text that sbBSC express higher levels of Bcl-2, which protects them from detachment-mediated apoptosis. To further support this conclusion, the authors should repeat their in vitro anoikis assay using sbBSC, which should express higher level of Bcl-2 and therefore be protected from apoptosis with anti-integrin  $\alpha 6$  Ab treatment. Additionally, they should add ABT-199 to their sbBSC to see if detachment-associated apoptosis is rescued by chemically inhibiting Bcl-2.

#### MINOR CONCERNS

1. All figure legends (Labels): In every figure legend, please indicate what each abbreviation stands for.
2. Fig.1 (Experiment): The authors demonstrate that Bcl-2 mRNA is more highly expressed in sbBSC compared to bBSC and n-B. It would be compelling to include Bcl-2 immunostaining to illustrate that Bcl-2 protein is localized to sbBSC.
3. Page 4 (Experiment detail): Include the age of the mice at the time of ABT-199 treatment and sample collection for the data in Fig. 1a.

4. Page 5 (Grammar): Section title should be changed to "Cyclic hair regeneration depends on suprabasal bulge SCs protected by Bcl-2".
5. Page 5 (Reference): Please include reference(s) to support that Lef1 and Runx1 are, "transcription factors associated with hair growth".
6. Page 6 (Claim regarding E.D. Fig 3c): Although "no significant changes in the expression of Lef1 and Runx1 were detected following ABT-199 treatment" with the sample size, there appears to be a clear upwards trend in expression of Lef1 and Runx1. This should be addressed in the text.
7. Page 6 (Claim regarding Fig. 1): The text claims that, "the data propose Bcl-2-mediated protection of sbBSCs solely during telogen"; however, there are no data that address if Bcl-2-mediated protection occurs during catagen. Therefore, the text should more accurately read, "the data propose Bcl-2-mediated protection of sbBSCs during telogen but not during anagen."
8. Fig. 2c (Statistics): The difference in Itga5 expression between bBSC and sbBSC appears dramatic, but it is not labeled as statistically significant with the sample size of n=3. The authors should state the what their "n" represents (mice?). Additionally, it seems likely that the difference would be statistically significant if the sample size is increased. if it is not feasible for the authors to add more samples, then it would be helpful for them to provide insight regarding why they believe the trend is not significant (e.g. sample variability).
9. Fig. 3a,b (Experiment detail): In the histology images of the control and Bcl-2EOE HF in catagen, it would be helpful to include lines to indicate how the height and width of the retracting epithelial strand was measured.
10. Page 8 (Reference): Please include reference(s) to support that Keratin 15, Sox 9 and Nfatc1 are, "...known BSC marker[s]..."
11. E.D. Fig. 6d (Reference): There is no explanation (in neither the main text nor the figure legend) for why P-cadherin staining was performed.
12. Fig. 4c,d (Experimental details): It would be helpful to include more information in the main text about the design of the BrdU experiment, e.g. the rationale behind the timing/length of the pulse and chase.
13. Page 9 (Claim): The claim, "Clearly, Bcl-2 expression interferes with HF regression by inhibiting cell loss and promoting cell survival, thereby increasing HF bulge and HG size and altering their architecture," should be modified to more accurately communicate that Bcl-2 overexpression throughout the HF/epidermis interferes with regression.
14. Page 10 (Experimental detail): In the sentence, "...we addressed the impact of Bcl-2 on epidermal tumour formation using the K15 $\Delta$ NLef1 mice that express a mutated form of the transcription factor Lef1 specifically in BSCs..." the authors should specify what kind of mutation (e.g. activating, knock down, knock out) these mice harbor.

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Referee #2:

In this manuscript by Geueke et al, titled "Differential requirement of Bcl-2-regulated cell death governs hair follicle stem cell function", the authors suggest that Bcl-2 expression in a specific subset of stem cells within the bulge region of hair follicles, i.e. suprabasal bulge stem cells (sbBSCs), as opposed to basal bulge stem cells (bBSCs), are essential for the cyclical regeneration of hair follicles mice. They argue that sbBSCs are particularly susceptible to anoikis and that Bcl-2 prevents Bim/Bmf-mediated apoptosis in this context. There is a quite a bit of literature that indicates a role for Bcl-2 and Bim in the maintenance of hair follicles (and pigmentation involving the survival of melanocytes), so the primary novelty of the current work emanates from the specific roles proposed for these proteins in sbBSCs. Unfortunately, in addition to a number of other concerns, this reviewer is simply not convinced that such specificity actually exists.

## Major criticisms:

1. The authors argue that there is a significant increase in active caspase-3 positive (aCasp3+) sbBSCs within the bulge following treatment of mice with Bcl-2 antagonist, ABT-199. Setting aside the fact that the presence of aCasp3 does not necessarily indicate that a cell is dying, this reviewer sees green cells outside the follicle but no convincing evidence that aCasp3+ cells are present within the bulge (Fig. 1A). The evidence in aCasp3+ cells in the follicles of tail skin (EV1e) is convincing but not in back skin (Fig. 1a).
2. The authors suggest that no depletion of sbBSCs was observed in Bcl-2<sup>EKO</sup> mice, compared to treatment with ABT. Thus, they conclude that acute inhibition of Bcl-2 is required to render these cells sensitive to anoikis. While this is certainly a possibility, they fail to note that ABT is NOT selective for Bcl-2, as it also readily inhibits Bcl-xL and to lesser extent other antiapoptotic Bcl-2 family members (excluding Mcl-1).
3. In the second half of the paper, the authors utilize overexpression of Bcl-2 using a K14Cre driver to express Bcl-2 in stem cells. However, since they are using a LSL-Bcl2-IRES-GFP, it is unclear why they have not stained for GFP to verify the location of Bcl-2 expression? One suspects that Bcl-2 expression is present in both sbBSCs and bBSCs, which make it impossible to make any specific claims about Bcl-2's selective role in sbBSCs.
4. The authors stain with antibodies to aCasp9 and aCasp8 and conclude that both the intrinsic and extrinsic pathways are activated during cell death. Caspase-8 however can be cleaved downstream of the intrinsic pathway via a casp9 --> casp3 --> casp6 --> casp8 feedback loop and the cleaved caspase-8 is not active in this context. If caspase-8 were activated and cleaved with a TNF/Fas/TRAIL receptor DISC, Bcl-2 would not prevent this cleavage.
5. All of the studies with Bcl-2 overexpression must be interpreted with caution, because Bcl-2 will of course inhibit apoptosis in essentially any cell type in which it is expressed. The accumulation of cells within the follicle can in turn skew RNA-seq results, etc. because it becomes unclear whether changes in gene expression are simply due the presence of cells that would normally not be present. It is similarly questionable whether one can conclude that, since Bcl-2 overexpression blocked apoptosis in BSCs from K15deltaNLef1 mice, downregulation of Bcl-2 in these same cells is therefore responsible for the death that would normally be observed. This explanation is certainly plausible, but Bcl-2 overexpression would almost certainly inhibit cell death regardless of the precipitating stimulus. Similarly, given that BSCs are known to be the targets of DMBA and give rise to skin cancer in chemically-induced skin carcinogenesis, it is entirely predictable that Bcl-2-mediated inhibition of apoptosis in treated BSCs would result in more tumors.

## Minor criticisms:

The authors should really consider providing more background information on normal hair follicle biology, particularly for a non-specialized journal. They should also consider adding more detail to the figure legends, reiterating the meaning of various abbreviations which are not immediately recognizable to a reader outside the field.

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Referee #3:

This work describes how epithelial stem cells-expressed bcl-2 affect cyclic hair follicle growth. The

author used ABT-199 as an antagonist for bcl-2 to assess the role of bcl-2 in hair regeneration. They showed that the lack of bcl-2 caused reduction in the expression of hair growth transcriptional factors such as Lef1 and Runx1, delaying anagen transition and hair graying. Moreover, they utilized Bcl-2EKO mice and observed apoptosis (anoikis) and loss of the supra-basal bulge stem cells (sbBSCs), indicating bcl-2 dependency of sbBSCs during telogen phase. In contrast, overexpression of bcl-2 within epidermis using Bcl-2EOE mice, blocked apoptosis and normal cell death in catagen, caused enlarged hair follicle bulge contain keratinocytes. Treatment of K15  $\Delta$ NLef1/Bcl-2EOE mice with DMBA progressed BSC-driven epidermal tumor formation and impaired transition of hair follicle from telogen to anagen phase. Collectively, this manuscript shows interesting new data dissecting the role for bcl-2 in bulge stem cell homeostasis and hair follicle regeneration. Figures are well organized and most images are beautiful. The study will be of interest for readers of this journal.

The authors mentioned "FACS analysis of BSCs revealed the specific loss of CD34+/ $\text{Itga6}^{\text{low}}$  BSCs upon ABT-199 treatment". It is not clear how long after treatment the authors have evaluated the BSCs? Please clarify.

The authors have only evaluated the expression of the bcl2 in sbBSCs and bBSCs in bulge. The authors need to also assess the expression of bcl-2 in the SHG, and need more clear descriptions of in which cell types BCL2 is expressed among non bulge stem cells.

The authors need to address the hair gray phenotype in this study resulted only from the depletion of sbBSCs. Some molecular mechanisms underlying this need to be discussed.

The purpose of the study need to be more explicitly stated in the introduction.

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Referee #4:

In this manuscript, Geueke et al., document that a differential Bcl-2-expression within the hair follicle bulge regulates an anoikis response in suprabasal bulge cells and governs HF regeneration. Using a pharmacological inhibitor and a genetic mouse model overexpressing Bcl-2 in the epidermis, the authors show that Bcl-2 function impacts on bulge organization, HFSC quiescence, and HF growth. Also, the authors observed that overexpression of Bcl-2 increases the incidence and frequency of skin tumors by promoting cell survival.

Even though this proposal is interesting, I found the data in the manuscript still preliminary, and not of sufficient depth to state some of the proposed conclusions about the role of Bcl-2 in the regulation of HFSC function to provide an advance to the field.

Major points:

1. The most intriguing aspect of the manuscript is the finding of a different response within HF bulge cell populations in governing HF regeneration. The authors document an increased expression of Bcl2 in suprabasal cells, thereby an increased sensitivity to Bcl-2 inhibition to undergo apoptosis. But how sbBSC cell death during telogen leads to a delay in HF regeneration?

The authors characterize total epidermal changes in Lef1 and Runx1 mRNA levels and document hair greying. Still, unfortunately, despite being the primary claim of the ms, the authors did not address how Bcl-2 regulated cell death governs the function of HFSC. Is it through deregulation of

basal BSC activation, e.g., premature differentiation, senescence, signaling?

2. Fig. 1. Is the Bcl-2-driven antiapoptotic response only relevant during the assessed competent telogen phase (P50) compared to the refractory and telogen-anagen transition stages?

3. The authors state, "mechanistically we demonstrate that an SC subpopulation undergoes anoikis during HF regeneration, thus explaining their strict Bcl-2 dependency." Unfortunately, the analyses are not of sufficient depth to reach those definite conclusions. On the one hand, the authors do not assess anoikis during HF regeneration, but in cell culture upon blocking integrin-mediated adhesion (Fig. 1d). On the other hand, their cell system does not mimic the presence of SC subpopulations, nor a differential expression of Bcl-2 expression. Finally, it is well-acknowledged that blocking integrin adhesion with antibodies leads to cell death in both Bcl-2 dependent and independent ways, how do the authors link this global event to their model in HF regeneration?

4. Page 7, last paragraph. The authors state, "our data show that Bcl-2 expression is essential to protect sbBSCs from detachment-induced apoptosis during telogen." In line with my previous comments, this has yet to be demonstrated. However, experiments in vitro can suggest it, does the overexpression of Bcl-2 protects basal cells in culture from cell death upon the addition of integrin blocking antibodies? What would be the effect of the inhibition of Bcl-2 under those conditions?

5. Using mouse genetics, the authors overexpressed Bcl-2 in K14 positive cells and focused on different aspects, ranging from catagen, telogen characterization, and tumorigenesis. Although interesting, from this point, the flow of the manuscript was disrupted from the main focus: the requirement of Bcl-2-regulated cell death driven regulation of HF stem cell function. In any event, I have some suggestions regarding those findings, starting from point 6 below.

6. Fig. 4. Regressing HFs that eventually retract give rise to telogen-like HF. Based on their abnormal morphology - are these rather cysts than bonafide telogen HF? The authors proposed, based on K15 expression levels, the presence of an enlarged bulge. Are there any changes in basal and suprabasal HFSC populations based on  $\alpha 6$  integrin High CD34+ versus  $\alpha 6$  integrin Low CD34+ levels?

7. Since K14-Bcl2 mouse skin exhibits abnormally enlarged telogen-like HF, without showing an increase in cell proliferation, the authors state: "Clearly, Bcl-2 expression interferes with HF regression by inhibiting cell loss", as a potential explanation to the increased cellularity. A lineage-tracing system is required to state those conclusions. Are any markers of HF layers expressed in the telogen-like structures? FACS analyses of defined populations and expression of markers through PCR evaluation can provide better insight into this aspect than analyzing the mRNA expression of markers in the epidermis.

8. A role for Bcl-2 and other family members in skin tumorigenesis has been previously documented, in particular under skin stress conditions and photo DNA damage. Do the authors aim to propose a role for Bcl-2 in protecting K15 tumor-initiating cells from cell death? Page 10, last sentence.

Although an exciting proposition, with the obtained results, it is not possible to reach those conclusions. The authors did not overexpress Bcl2 specifically in BSC but by using a K14 mouse model. The observed effect could be indirect, through signals arising from neighboring cells overexpressing Bcl2. However, control and CD34BclE2OE cells can be isolated and transplanted to compare their efficiency in tumor formation, survival, and proliferation.

9. Fig. 5c. The authors quantified the number of aCas3+ cells, are there any differences in cell proliferation or differentiation between the different systems?

10. Fig. 5. The authors should show if there are any differences in apoptosis and cell proliferation in the tumors shown in Fig 5g.

11. The authors indicate that no changes in tumor type or sebaceous differentiation occur in their model compared to controls. However, in Extended Data Figure 7d, Scd1 expression levels differ from those observed in controls.

Minor points.

1. Page 4. The sentence "a continuous turnover and apoptosis of HG cells" is not clear. Please explain.

2. Extended data figure 1f. The pictures show a high level of aCas3+ in the HG upon ABT-199 treatment. The representative image differs from the graph, where no significant differences in HG aCas3+ cells are shown.

3. K14Cre- Rosa26LSL.Bcl2.IRES.GFP. A better characterization of the model is missing. What is the ratio of Bcl-2 increase compared to controls? Is Bcl-2 evenly expressed in K14 positive cells, or is there any mosaicism present in their system?

4. K14Cre- Rosa26LSL.Bcl2.IRES.GFP. The authors document an opposite response during catagen to the one observed previously using another K14 Bcl-2 mouse model, which instead led to an accelerated catagen progression (Muller-Rover et al. 1999). How do the authors reconcile the differences?

5. I believe reorganizing the manuscript could benefit the flow of ideas. For example, first, show the RNA seq analyses as a tool to address functional heterogeneity between basal and suprabasal cells. Second, focus on the identified cell death and survival pathways in suprabasal cells. Next, show the differential response of sbBSCs to the Bcl-2 inhibitor, to later focus on the potential mechanisms leading to the control of HF regeneration, etc.

**Response to the Reviewers:****Geueke et al.**

point-by-point response

**Referee #1:**

This manuscript by Geueke et al. describes the novel finding that Bcl-2 is specifically expressed in a subpopulation of hair follicle bulge stem cells, and plays an important role in the regulation of hair follicle cycling. It was previously known that Bcl-2 promotes cell survival by blocking apoptosis, is important for the survival of skin melanocytes, and contributes to the development of some cancers, including B cell lymphomas. This paper provides new insight into the importance of Bcl-2 for the maintenance of suprabasal bulge stem cells (sbBSC) in hair follicles, which are important for regulating the hair regrowth cycle. Additionally, the authors demonstrate the consequences of aberrant activation of Bcl-2 in the epidermis: complete inhibition of caspase 3-mediated apoptosis, impair hair regrowth, and aggravated development of epidermal tumors. Overall, the authors carefully state the major conclusions from their data. Below, I suggest a few additional experiments to strengthen their proposed mechanism that Bcl-2 protects sbBSC from anoikis, which I think is currently their weakest claim. I also suggest changes to the text to make the manuscript clearer and stronger.

The authors would like to thank the reviewer for his/her supportive comments and valuable suggestions, which helped to substantially improve the overall quality of our study. Encouraged by the reviewer's statements we have revised the manuscript.

Our specific comments to the issues raised by reviewer #1 are outlined below.

**MAJOR CONCERNS**

1. Clarity of experimental timing: Overall, this manuscript would benefit from more consistent and explicit description of the timing of each experiment. The authors probe several different timepoints during development and hair cycling, but do not consistently provide clear justification for their experimental design. Providing this detail will help readers better understand the precise scientific question the authors are addressing with each experiment, and the significance of the results.

We have revised the text of the manuscript to better explain the experimental design and provide clear justification for the experimental set up. To provide a more consistent and explicit description, we added schemes to the figures (Fig 1A,C; Fig. 2B,C; Fig. EV1C, Appendix Fig. S2B,C) and included detailed information on the specific time points of experiments within the figure legends.

2. Fig. 2d (Experiment): While the concept of this *in vitro* anoikis assay is logical, critical controls and/or references are missing. The data presented show that aCas3+ staining increases when cultures are treated with an integrin  $\alpha 6$ -blocking antibody compared to untreated controls. However, it is not shown (or referenced) that this antibody induces keratinocyte detachment. Additionally, without an Ig control, it cannot be discerned whether this effect is caused by blocking integrin  $\alpha 6$ , or merely by the presence of antibody.

Given that the cultivation of sbBSC population is not reliable in the *in vitro* 3D model system (Chacon-Martinez et al. 2017), we now removed these initial studies and revised the figures and text pointing out that our RNA-seq results suggest a mechanism of anoikis-like cell death of sbBSCs. The authors realized that some of the conclusions might have been overstated and, therefore, revised the text to better reflect the main observations and data interpretation.

3. Fig. 2 (Experiment): The authors claim in the text that sbBSC express higher levels of Bcl-2, which protects them from detachment-mediated apoptosis. To further support this conclusion, the authors should repeat their *in vitro* anoikis assay using sbBSC, which should express higher level of Bcl-2 and therefore be protected from apoptosis with anti-integrin  $\alpha 6$  Ab treatment. Additionally, they should add ABT-199 to their sbBSC to see if detachment-associated apoptosis is rescued by chemically inhibiting Bcl-2.

As discussed above (major point 2), cultivation of sbBSC population is not consistent in the *in vitro* 3D model system (Chacon-Martinez et al. 2017). We thus removed these initial studies and revised the figures and text indicating that our RNA-seq results suggest a mechanism of detachment-induced apoptosis of sbBSCs.

#### MINOR CONCERNS

1. All figure legends (Labels): In every figure legend, please indicate what each abbreviation stands for.

We have revised the figure legends accordingly.

2. Fig.1 (Experiment): The authors demonstrate that Bcl-2 mRNA is more highly expressed in sbBSC compared to bBSC and n-B. It would be compelling to include Bcl-2 immunostaining to illustrate that Bcl-2 protein is localized to sbBSC.

We extensively tried to detect endogenous Bcl-2 protein in mouse skin by immunofluorescence staining and immune histochemistry. In contrast to Bcl-2<sup>EOE</sup> mice that exhibit robust Bcl-2 protein, no Bcl-2<sup>+ve</sup> cells were detected in skin of wild-type mice due to either low antibody sensitivity or low endogenous protein levels hindering its detection (new Appendix Fig. S4). Our data point to later since quantitative western blot experiments showed robust Bcl-2 detection in Bcl-2<sup>EOE</sup> mice but weak signal in wildtype control animals.

3. Page 4 (Experiment detail): Include the age of the mice at the time of ABT-199 treatment and sample collection for the data in Fig. 1a.

We have included a new scheme in Figure 1A providing further experimental details on the specific time points of treatment and the age of mice.

4. Page 5 (Grammar): Section title should be changed to "Cyclic hair regeneration depends on suprabasal bulge SCs protected by Bcl-2".

The text has been revised.

5. Page 5 (Reference): Please include reference(s) to support that Lef1 and Runx1 are, "transcription factors associated with hair growth".

We have included the references for both transcription factors.

6. Page 6 (Claim regarding E.D. Fig 3c): Although "no significant changes in the expression of Lef1 and Runx1 were detected following ABT-199 treatment" with the sample size, there appears to be a clear upwards trend in expression of Lef1 and Runx1. This should be addressed in the text.

We have changed the text of the manuscript by explaining the variability in Lef1 and Runx1 expression level during anagen phase between the individual animals tested in this experiment. The histological analysis of mice shows clearly that all hair follicles are in anagen. Therefore, no changes in Lef1 and Runx1 expression between treated and control mice are expected.

7. Page 6 (Claim regarding Fig. 1): The text claims that, "the data propose Bcl-2-mediated protection of sbBSCs solely during telogen"; however, there are no data that address if Bcl-2-

mediated protection occurs during catagen. Therefore, the text should more accurately read, "the data propose Bcl-2-mediated protection of sbBSCs during telogen but not during anagen."  
[We have made textual changes to clearly state that telogen and anagen hair cycle phases were investigated.](#)

8. Fig. 2c (Statistics): The difference in Itga5 expression between bBSC and sbBSC appears dramatic, but it is not labeled as statistically significant with the sample size of n=3. The authors should state the what their "n" represents (mice?). Additionally, it seems likely that the difference would be statistically significant if the sample size is increased. if it is not feasible for the authors to add more samples, then it would be helpful for them to provide insight regarding why they believe the trend is not significant (e.g. sample variability).

[As stated in our response to major points 2 and 3 we have removed this data due to technical limitation of the \*in vitro\* system and to avoid any overstatement.](#)

9. Fig. 3a,b (Experiment detail): In the histology images of the control and Bcl-2EOE HF in catagen, it would be helpful to include lines to indicate how the height and width of the retracting epithelial strand was measured.

[To show in more detail how the retracting epithelial strand was measured, we have included the information applying state-of-the-art microscopy software to images in new Appendix Fig. S6A.](#)

10. Page 8 (Reference): Please include reference(s) to support that Keratin 15, Sox 9 and Nfatc1 are, "...known BSC marker<s>..."

[We included the references in the revised version of our manuscript.](#)

11. E.D. Fig. 6d (Reference): There is no explanation (in neither the main text nor the figure legend) for why P-cadherin staining was performed.

[We have made textual changes to explain that P-cadherin staining was performed to analyse HG cells.](#)

12. Fig. 4c,d (Experimental details): It would be helpful to include more information in the main text about the design of the BrdU experiment, e.g. the rationale behind the timing/length of the pulse and chase.

[We have included the experimental details on the BrdU experiment assay as measurement for proliferation in the tissue \(one pulse and 1h chase\) in the revised version of the manuscript.](#)

13. Page 9 (Claim): The claim, "Clearly, Bcl-2 expression interferes with HF regression by inhibiting cell loss and promoting cell survival, thereby increasing HF bulge and HG size and altering their architecture," should be modified to more accurately communicate that Bcl-2 overexpression throughout the HF/epidermis interferes with regression.

[We have changed the text now stating "Bcl-2 overexpression throughout the HF/epidermis interferes with regression".](#)

14. Page 10 (Experimental detail): In the sentence, "...we addressed the impact of Bcl-2 on epidermal tumour formation using the K15 $\Delta$ NLef1 mice that express a mutated form of the transcription factor Lef1 specifically in BSCs..." the authors should specify what kind of mutation (e.g. activating, knock down, knock out) these mice harbor.

[We have included more details on the mutant Lef1 used in our study in the revised version of our manuscript explaining the N-terminal deletion and dominant negative function of this particular Lef1 construct.](#)

point-by-point response

**Referee #2:**

In this manuscript by Geueke et al, titled "Differential requirement of Bcl-2-regulated cell death governs hair follicle stem cell function", the authors suggest that Bcl-2 expression in a specific subset of stem cells within the bulge region of hair follicles, i.e. suprabasal bulge stem cells (sbBSCs), as opposed to basal bulge stem cells (bBSCs), are essential for the cyclical regeneration of hair follicles mice. They argue that sbBSCs are particularly susceptible to anoikis and that Bcl-2 prevents Bim/Bmf-mediated apoptosis in this context. There is a quite a bit of literature that indicates a role for Bcl-2 and Bim in the maintenance of hair follicles (and pigmentation involving the survival of melanocytes), so the primary novelty of the current work emanates from the specific roles proposed for these proteins in sbBSCs. Unfortunately, in addition to a number of other concerns, this reviewer is simply not convinced that such specificity actually exists.

We thank this reviewer for his/her critical view and would like to ask him/her to reconsider our quite striking observations using ABT-199. Our detailed investigations identified sbBSCs as the main SC compartment with a marked susceptibility to ABT-199 and subsequent analyses identified Bim/Bmf as the possible mediator of mitochondrial apoptosis in BSCs. By using conditional Bcl-2 knock-in and knock-out mice, our data conclusively supported the results obtained by ABT-199. We do agree that some previous studies already indicated the involvement of Bcl-2 in mammalian epidermis, but none of them studied the effects of ABT-199 in skin SC compartment nor described the selectivity of Bcl-2 actions in sbBSCs. We also made textual changes to better explain the rationale behind the Bcl-2 overexpression experiments.

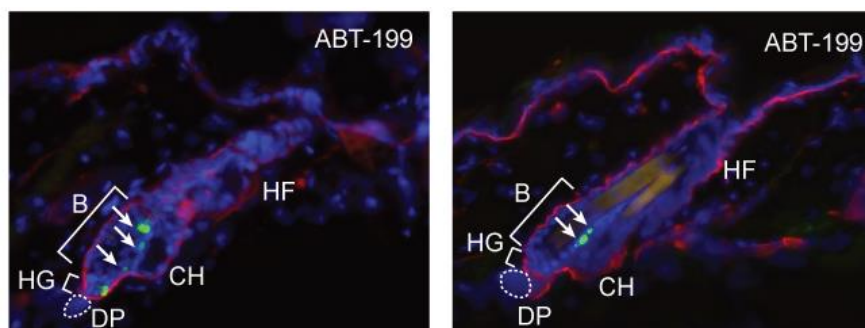
Our specific comments to the concerns raised by reviewer #2 are outlined below.

Major criticisms:

1. The authors argue that there is a significant increase in active caspase-3 positive (aCasp3+) sbBSCs within the bulge following treatment of mice with Bcl-2 antagonist, ABT-199. Setting aside the fact that the presence of aCasp3 does not necessarily indicate that a cell is dying, this reviewer sees green cells outside the follicle but no convincing evidence that aCasp3+ cells are present within the bulge (Fig. 1A). The evidence in aCasp3+ cells in the follicles of tail skin (EV1e) is convincing but not in back skin (Fig. 1a).

We thank this reviewer for his/her discerning view. In our opinion, the discrepancy in the number aCasp3<sup>+</sup> cells between tail and back skin is attributed to the whole mounts (tail epidermis) vs. sections (backskin) and the different techniques used to isolate and analyse the tissue (microscopy of section vs. 3D z-stack of HF structures).

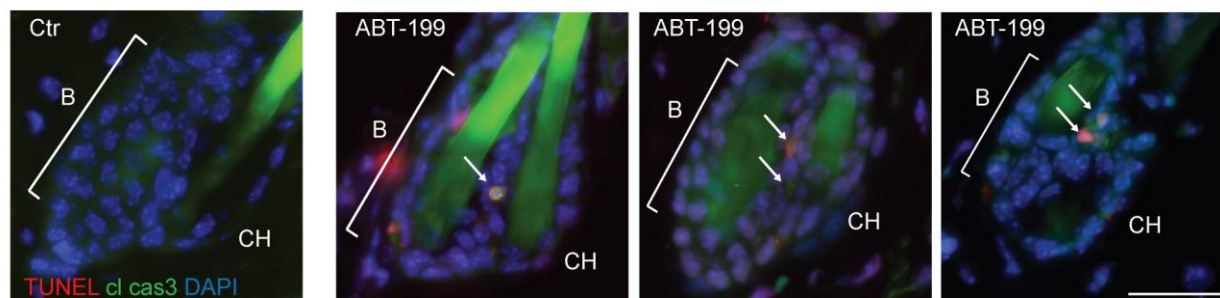
Below, we present two additional images of ABT-199 treated back skin samples to demonstrate the specific signal for aCasp3<sup>+</sup> apoptotic keratinocytes within the sbBSCs. In addition to the immunostainings for aCasp3, analyses of the HF bulge composition by FACS indicated the specific loss of the sbBSC population from back skin of ABT-199 treated mice (please see also Fig. 1 C,D).



*additional Figure 1: Apoptosis of sbBSCs following treatment with ABT-199.*

aCas3 immunostaining (green), integrin6 (red) and DAPI (blue) in backskin samples of ABT-199 treated mice. Please note specific signal for aCas3 in sbBSCs (arrows). B: bulge; CH: club hair; DP: dermal papilla; HF: hair follicle; HG: secondary hair germ.

Immunostaining for activated caspase 3 usually represents the ultimate step or the 'point-of-no-return' of apoptosis and is frequently used as the 'state of the art' method for detecting dying/apoptotic cells in different tissues including the skin. Independently, we also confirmed the specific cell death of sbBSC in ABT-199 treated mice by Tunnel staining showing apoptotic cells double positive for aCas3 and Tunnel (see additional Figure 2 below).



*additional Figure 2: Apoptosis of sbBSCs following systemic treatment with ABT-199.*

aCas3 immunostaining (green), Tunnel (red) and DAPI (blue) in backskin samples of ABT-199 treated mice. Please note double positive sbBSCs (aCas3 and Tunnel, arrows). B: bulge; CH: club hair; DP: dermal papilla; HF: hair follicle; HG: secondary hair germ. Please note Tunnel<sup>+</sup> cells in sb bulge and double positive apoptotic sbBSCs.

2. The authors suggest that no depletion of sbBSCs was observed in Bcl-2<sup>EKO</sup> mice, compared to treatment with ABT. Thus, they conclude that acute inhibition of Bcl-2 is required to render these cells sensitive to anoikis. While this is certainly a possibility, they fail to note that ABT is NOT selective for Bcl-2, as it also readily inhibits Bcl-xL and to lesser extent other antiapoptotic Bcl-2 family members (excluding Mcl-1).

This recommendation is very well taken. According to the previous data (Sours et al. Nature medicine 2013) demonstrating high specificity of ABT-199 toward Bcl-2 ( $BCL-2: K_i < 0.010 \text{ nM}$ ;  $BCL-X_L: K_i = 48 \text{ nM}$ ) we initially presumed Bcl-2 selectivity of ABT-199 in our studies. Although this view is commonly accepted, we cannot rule out the possibility that this inhibitor might in addition interfere with other Bcl-2 proteins, which might also apply for every chemical compound treatment *in vivo*. As this reviewer recommended, we now included this possibility as an explanation for the discrepancy between the ABT-199 treatment and the Bcl-2 knockout mice to our discussion. Independently, we would like to mention that in contrast to Bcl-2, Bcl-xL is not differently expressed in sbBSCs compared to bBSC and non-stem cells as demonstrated in new Appendix Fig. S1A.

3. In the second half of the paper, the authors utilize overexpression of Bcl-2 using a K14Cre driver to express Bcl-2 in stem cells. However, since they are using a LSL-Bcl2-IRES-GFP, it is unclear why they have not stained for GFP to verify the location of Bcl-2 expression? One suspects that Bcl-2 expression is present in both sbBSCs and bBSCs, which make it impossible to make any specific claims about Bcl-2's selective role in sbBSCs.

We have included qRT-PCR and images for Bcl-2 expression and GFP detection in the back skin and epidermal tail whole mounts from Bcl-2<sup>EOE</sup> mice (LSL-Bcl2-IRES-GFP x K14Cre) in the revised version of our manuscript. These results indicate robust expression of Bcl-2 in all K14<sup>+</sup> basal keratinocytes in HFs and the interfollicular epidermis and their progeny (new Appendix Fig. S4B-E).

We agree with the reviewer that Bcl-2, as expected, is present in both, bBSCs and sbBSCs in Bcl-2<sup>EOE</sup> mice. Accordingly, changes in BSC function/architecture might not solely arise from the sbBSC subpopulation. We would like to point out that the data showing a role for anti-apoptotic proteins in sbBSCs emanate from the ABT-199 treatment studies and not from the analysis of Bcl-2 overexpressing mice. We have revised the text of the manuscript to better explain this issue and the rationale for generating the Bcl-2 overexpressing mouse model. In particular, we aimed to specifically test if Bcl-2 overexpression (and potential block in apoptosis) plays an instructive role in BSCs and is able to change composition and function of BSCs *in vivo* (new Fig. 4C) and to investigate Bcl-2 for SC function during hair regeneration and tumour formation (new Fig. EV4A-D).

4. The authors stain with antibodies to aCasp9 and aCasp8 and conclude that both the intrinsic and extrinsic pathways are activated during cell death. Caspase-8 however can be cleaved downstream of the intrinsic pathway via a casp9 --> casp3 --> casp6 --> casp8 feedback loop and the cleaved caspase-8 is not active in this context. If caspase-8 were activated and cleaved with a TNF/Fas/TRAIL receptor DISC, Bcl-2 would not prevent this cleavage.

We thank this reviewer for this critical point and rephrased our explanation regarding the stainings of caspases. Notably, the mechanistic details about caspases were not the main focus of our manuscript and rather will be investigated in a future project. Nonetheless, our immunofluorescence data show that antibodies against aCaspase-3 and aCaspase-9 detect the same hair follicle population during catagen and thus support our conclusion that aCaspase-3 staining indeed marks apoptotic cells (see also point 1).

5. All of the studies with Bcl-2 overexpression must be interpreted with caution, because Bcl-2 will of course inhibit apoptosis in essentially any cell type in which it is expressed. The accumulation of cells within the follicle can in turn skew RNA-seq results, etc. because it becomes unclear whether changes in gene expression are simply due the presence of cells that would normally not be present. It is similarly questionable whether one can conclude that, since Bcl-2 overexpression blocked apoptosis in BSCs from K15 $\Delta$ NLef1 mice, downregulation of Bcl-2 in these same cells is therefore responsible for the death that would normally be observed. This explanation is certainly plausible, but Bcl-2 overexpression would almost certainly inhibit cell death regardless of the precipitating stimulus. Similarly, given that BSCs are known to be targets of DMBA and give rise to skin cancer in chemically-induced skin carcinogenesis, it is entirely predictable that Bcl-2-mediated inhibition of apoptosis in treated BSCs would result in more tumors.

We thank this reviewer for this very delicate point and we completely agree with the caution in the interpreting the data obtained by Bcl-2 knock-in. We first would like to mention that our RNAseq data were carried out in wild type mice without Bcl-2 overexpression. As also acknowledged by this reviewer, our Bcl-2 overexpression experiment and genetic “rescue” of the down-regulation of Bcl-2 in K15 $\Delta$ NLef1 mice provide a plausible explanation for the essential role of Bcl-2 in this context. In this regard, our previous analysis of other pro- and anti-apoptotic

molecules pointed to significant changes only in SC-specific Bcl-2 expression upon mutLef1 expression (Petersson et al., 2015). To avoid any overstatement of these conclusions, we added this information and revised the text of the manuscript by deleting the following statement: “Apoptosis was efficiently blocked in K15ΔNLef1/Bcl-2<sup>EOE</sup> mice, thus indicating that down-regulation of Bcl-2 in BSCs is the reason for apoptosis in K15ΔNLef1 mice”.

Independently, the possible role of apoptosis for tumorigenesis is indeed predictable; however, the experiment and the data show the actual outcome and provide solid evidence for it. This is particularly relevant for the K15ΔNLef1 mice, where the block of apoptosis by Bcl-2 overexpression is accompanied by a concomitant block in the proliferation of HF BSCs, suggesting the involvement of apoptosis-induced proliferation (AiP), a process that has been described in other tissues. Importantly, our data suggest the accumulation of (mutated) SCs and not proliferation *per se* as the driver for tumour formation in K15ΔNLef1 mice. We have revised the text and included the proliferation data (new Fig. 6D,E) to clarify our observations.

#### Minor criticisms:

The authors should really consider providing more background information on normal hair follicle biology, particularly for a non-specialized journal. They should also consider adding more detail to the figure legends, reiterating the meaning of various abbreviations which are not immediately recognizable to a reader outside the field.

As the reviewer suggested, we provide more background information as well as details in the figure legends in the current version of our manuscript.

point-by-point response

**Referee #3:**

This work describes how epithelial stem cells-expressed bcl-2 affect cyclic hair follicle growth. The author used ABT-199 as an antagonist for bcl-2 to assess the role of bcl-2 in hair regeneration. They showed that the lack of bcl-2 caused reduction in the expression of hair growth transcriptional factors such as Lef1 and Runx1, delaying anagen transition and hair graying. Moreover, they utilized Bcl-2EKO mice and observed apoptosis (anoikis) and loss of the supra-basal bulge stem cells (sbBSCs), indicating bcl-2 dependency of sbBSCs during telogen phase. In contrast, overexpression of bcl-2 within epidermis using Bcl-2EOE mice, blocked apoptosis and normal cell death in catagen, caused enlarged hair follicle bulge contain keratinocytes. Treatment of K15  $\Delta$ NLef1/Bcl-2EOE mice with DMBA progressed BSC-driven epidermal tumor formation and impaired transition of hair follicle from telogen to anagen phase. Collectively, this manuscript shows interesting new data dissecting the role for bcl-2 in bulge stem cell homeostasis and hair follicle regeneration. Figures are well organized and most images are beautiful. The study will be of interest for readers of this journal.

The authors like to thank the reviewer for his/her positive evaluation and encouraging comments. Our response to the points raised by the reviewer are outlined below.

The authors mentioned "FACS analysis of BSCs revealed the specific loss of CD34<sup>+</sup>/Itga6<sup>low</sup> BSCs upon ABT-199 treatment". It is not clear how long after treatment the authors have evaluated the BSCs? Please clarify.

Based on the reviewers comment we have now included the experimental details in the figures (additions to Fig. 1A,C; Fig. EV1C).

The authors have only evaluated the expression of the bcl2 in sbBSCs and bBSCs in bulge. The authors need to also assess the expression of bcl-2 in the sHG, and need more clear descriptions of in which cell types BCL2 is expressed among non bulge stem cells.

To address this point we have performed additional experiments analysing the expression of Bcl-2 in the secondary hair germ (sHG) following sorting of sHG cells (Lgr5<sup>+</sup>/CD34<sup>-</sup> cells; Jaks et al. Nat Genet 2008) (Appendix Fig. S1B). The data show that sorted cells are positive for the HG marker P-cadherin and express slightly less Bcl-2 compared to BSCs (Appendix Fig. S1B). Further, we have analysed Lrig1<sup>+</sup> SCs of the junctional zone following sorting of GFP<sup>+</sup> keratinocytes from epidermis of Lrig1Cre-eGFP mice (Page et al. Cell Stem Cell 2013). As expected, GFP<sup>+</sup> cells express Lrig1 and are negative for the BSC marker CD34. Our data reveal that SC of the JZ express less Bcl-2 than BSCs (Appendix Fig. S1C). Together our data demonstrate that Bcl-2 is expressed at very low level outside the BSC with sbBSCs showing the highest expression.

The authors need to address the hair gray phenotype in this study resulted only from the depletion of sbBSCs. Some molecular mechanisms underlying this need to be discussed.

The hair greying phenotype results from depletion of Bcl-2-protected melanocytes as shown previously by conventional Bcl-2 knock out studies (Kamada et al. 1995; Veis et al. 1993; Yamamura et al. 1996) and is thus independent from sbBSC depletion following ABT-199 treatment. We made textual changes to make this point more clear in the current version of the manuscript.

The purpose of the study need to be more explicitly stated in the introduction.

Based on this suggestion we have now revised the text of the manuscript to better explain the rationale of our study.

point-by-point response

**Referee #4:**

Comments for the authors:

In this manuscript, Geueke et al., document that a differential Bcl-2-expression within the hair follicle bulge regulates an anoikis response in suprabasal bulge cells and governs HF regeneration. Using a pharmacological inhibitor and a genetic mouse model overexpressing Bcl-2 in the epidermis, the authors show that Bcl-2 function impacts on bulge organization, HFSC quiescence, and HF growth. Also, the authors observed that overexpression of Bcl-2 increases the incidence and frequency of skin tumors by promoting cell survival.

Even though this proposal is interesting, I found the data in the manuscript still preliminary, and not of sufficient depth to state some of the proposed conclusions about the role of Bcl-2 in the regulation of HFSC function to provide an advance to the field.

We thank the reviewer for his/her comments, insightful questions and helpful suggestions. We have performed additional experiments addressing most of the major points and included these new data in the revised version of the manuscript. We realized that some of our conclusions might have been overstated and, therefore, revised the text to better reflect our main observations and data interpretation. The authors believe that after significant additions to the manuscript, our submission will be of sufficient depth to provide an advance to the field.

Our point-by-point response to the issues raised by the reviewer is outlined below.

Major points:

1. The most intriguing aspect of the manuscript is the finding of a different response within HF bulge cell populations in governing HF regeneration. The authors document an increased expression of Bcl2 in suprabasal cells, thereby an increased sensitivity to Bcl-2 inhibition to undergo apoptosis. But how sbBSC cell death during telogen leads to a delay in HF regeneration?

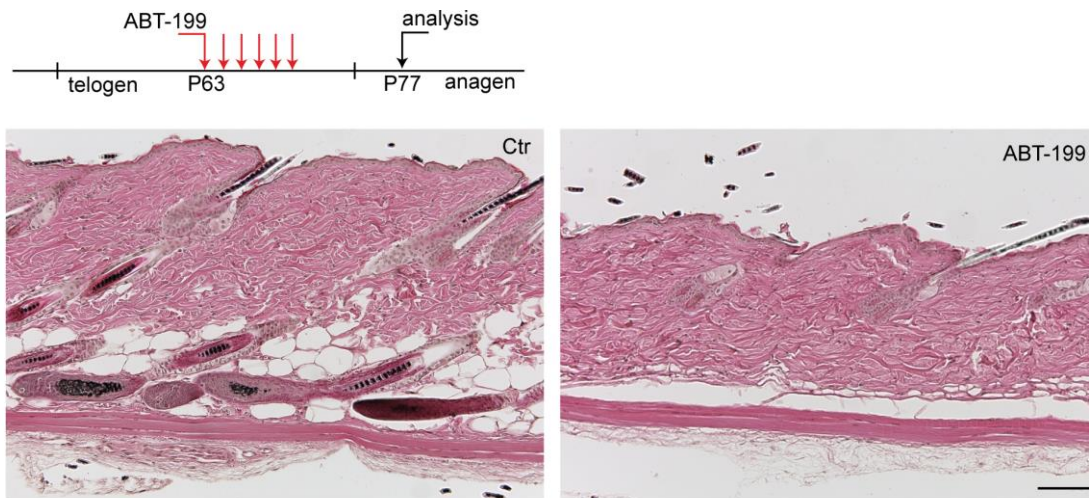
The authors characterize total epidermal changes in Lef1 and Runx1 mRNA levels and document hair greying. Still, unfortunately, despite being the primary claim of the ms, the authors did not address how Bcl-2 regulated cell death governs the function of HFSC. Is it through deregulation of basal BSC activation, e.g., premature differentiation, senescence, signaling?

The authors like to thank the reviewer for these interesting comments. The question raised by the reviewer '*how Bcl-2 regulated cell death governs the function of HFSC*' is a wide-open mechanistic question, which encompasses a bigger question about the physiological role of sbBSCs in hair follicle maintenance that is not directly addressed by the current study. Based on our data, we propose that the abrupt loss of sbBSCs results in a disruption of the normal architecture of the bulge SC compartment and thus impacts on the physiological function of BSCs. In this context, the hypothesis that sbBSC directly affect bBSC via an intra-bulge SC crosstalk is interesting and appealing and is now discussed in the manuscript. A direct contribution of sbBSCs to hair follicle growth during anagen may be an interesting possibility. However, we would like to point out that lineage tracing experiments addressing this issue are currently not possible due to the lack of experimental tools, e.g. sbBSC specific Cre lines for lineage tracing of sbBSC.

As we discuss in the manuscript, the hair greying phenotype is most likely not a direct consequence of sbBSC loss because it has been shown that Bcl-2 is required for melanocyte function (Kamada et al. 1995; Veis et al. 1993; Yamamura et al. 1996) and thus, the effect of ABT-199 treatment on hair greying may not involve HF bulge SCs *per se*.

2. Fig. 1. Is the Bcl-2-driven antiapoptotic response only relevant during the assessed competent telogen phase (P50) compared to the refractory and telogen-anagen transition stages?

To address this question, we have performed additional ABT-199 treatment experiments. In addition to blocking Bcl-2 during the refractory telogen phase (P50) as demonstrated in the manuscript (Fig. 1 and 2, Fig. EV1C-D), we also blocked Bcl-2 later during the competent phase (P63 onwards). These data are shown in the new figure below and reveal that ABT-199 treatment blocks subsequent anagen entry regardless of the time of treatment during both, the refractory and competent telogen.



additional Figure: Blocking Bcl-2 during the competent telogen phase inhibits anagen entry of hair follicles.

ABT-199 treatment starting at P63 during the competent stage of telogen hair follicles blocks subsequent anagen compared to control mice.

3. The authors state, "mechanistically we demonstrate that an SC subpopulation undergoes anoikis during HF regeneration, thus explaining their strict Bcl-2 dependency." Unfortunately, the analyses are not of sufficient depth to reach those definite conclusions. On the one hand, the authors do not assess anoikis during HF regeneration, but in cell culture upon blocking integrin-mediated adhesion (Fig. 1d). On the other hand, their cell system does not mimic the presence of SC subpopulations, nor a differential expression of Bcl-2 expression. Finally, it is well-acknowledged that blocking integrin adhesion with antibodies leads to cell death in both Bcl-2 dependent and independent ways, how do the authors link this global event to their model in HF regeneration?

We agree with the reviewer that it is not experimentally possible to assess anoikis during HF regeneration under physiological conditions *in vivo*. Furthermore, given that the cell culture system is limited with regard to the distribution of BSC subpopulations, we have now removed the cell culture experiments and tuned down our conclusion following transcriptome analysis by stating that the RNA-seq results suggest a detachment-induced cell death mechanism.

4. Page 7, last paragraph. The authors state, "our data show that Bcl-2 expression is essential to protect sbBSCs from detachment-induced apoptosis during telogen." In line with my previous comments, this has yet to be demonstrated. However, experiments *in vitro* can suggest it, does the overexpression of Bcl-2 protects basal cells in culture from cell death upon the addition of integrin blocking antibodies? What would be the effect of the inhibition of Bcl-2 under those conditions?

We agree with the reviewer that the physiological relevance of the Bcl-2-dependent anoikis for

BSCs is difficult to experimentally address *in vivo* (please see also our response to major point 3). Although the upregulation of anoikis-associated marker expression is detected specifically in sorted sbBSCs, other mechanistic possibilities may be considered. The authors realized that some of the conclusions might have been overstated and, therefore, revised the text to better reflect the main observations and data interpretation.

The reviewer is correct by pointing out that cultivation of sbBSC population is difficult in the *in vitro* 3D model system (Chacon-Martinez et al. 2017). We thus removed these initial studies and revised the figures and text pointing out that our RNA-seq results suggest such a mechanism.

5. Using mouse genetics, the authors overexpressed Bcl-2 in K14 positive cells and focused on different aspects, ranging from catagen, telogen characterization, and tumorigenesis. Although interesting, from this point, the flow of the manuscript was disrupted from the main focus: the requirement of Bcl-2-regulated cell death driven regulation of HF stem cell function. In any event, I have some suggestions regarding those findings, starting from point 6 below.

We have rearranged the text and figures for a better “flow of the manuscript”. In particular, we changed the order of Bcl-2<sup>EOE</sup> experiments to explain the rationale for Bcl-2 overexpression in the context of the potential instructive role of Bcl-2 and apoptosis regulation for the composition of the BSC compartment. Furthermore, we added new data specifically overexpressing Bcl-2 within HF BSCs (new Fig. EV4A-D). Please see our response below (major point 6).

6. Fig. 4. Regressing HF that eventually retract give rise to telogen-like HF. Based on their abnormal morphology - are these rather cysts than bonafide telogen HF? The authors proposed, based on K15 expression levels, the presence of an enlarged bulge. Are there any changes in basal and suprabasal HFSC populations based on a6 integrin High CD34+ versus a6 integrin Low CD34+ levels?

Based on the reviewer’s suggestions, we have analysed the abnormal morphology of HF in Bcl-2<sup>EOE</sup> mice after catagen in more detail. As presented in Fig. 4A, Fig. EV2A-D, new Appendix Fig. S5 and S6, we do not identify any epidermal cyst formation in Bcl-2<sup>EOE</sup> mice and show that the abnormal HF are *bona fide* telogen HF. Due to the block of apoptosis during catagen, our data suggest that in addition to HFSCs, the enlarged bulge area also consists of ORS keratinocytes and SC progeny that are normally lost during the regression phase of the hair cycle. In addition, we did not detect changes in sbBSC and bBSC as shown in new Fig. 4C in the revised manuscript.

7. Since K14-Bcl2 mouse skin exhibits abnormally enlarged telogen-like HF, without showing an increase in cell proliferation, the authors state: “Clearly, Bcl-2 expression interferes with HF regression by inhibiting cell loss”, as a potential explanation to the increased cellularity. A lineage-tracing system is required to state those conclusions. Are any markers of HF layers expressed in the telogen-like structures? FACS analyses of defined populations and expression of markers through PCR evaluation can provide better insight into this aspect than analyzing the mRNA expression of markers in the epidermis.

Previous publications have shown that mainly basal epithelial cells of HF (ORS) undergo apoptosis during catagen and that the differentiated inner layers of the hair follicle are lost by upward terminal differentiation (Mesa et al. 2016). Our data show that these HF keratinocytes are prevented from undergoing apoptosis during catagen in Bcl-2<sup>EOE</sup> mice (Fig. 5C,D; Appendix Fig. S6B,C; Fig. EV3D). Based on the question raised by the reviewer, we have performed immunofluorescence staining and qRT-PCR analysis of markers for HF layers. As presented in new Appendix Fig. S5A-D, these studies show no expression of HF lineage markers in deformed “telogen” HF revealing that no differentiated hair keratinocytes contribute to the enlarged bulge area. Our data show that Bcl-2 expression blocks apoptosis specifically in the retracting epithelial strand during catagen (Fig. 5C,D). Given the observation that no increase in proliferation is detected in Bcl-2<sup>EOE</sup> mice (Fig. EV3C), we thus conclude that the survival of basal

HF keratinocytes (ORS cells) that are normally lost during catagen contributes to the increased cellularity in “telogen” HF in Bcl-2<sup>EOE</sup> mice. We revised the text to clarify our data.

8. A role for Bcl-2 and other family members in skin tumorigenesis has been previously documented, in particular under skin stress conditions and photo DNA damage. Do the authors aim to propose a role for Bcl-2 in protecting K15 tumor-initiating cells from cell death? Page 10, last sentence.

Although an exciting proposition, with the obtained results, it is not possible to reach those conclusions. The authors did not overexpress Bcl2 specifically in BSC but by using a K14 mouse model. The observed effect could be indirect, through signals arising from neighboring cells overexpressing Bcl2. However, control and CD34BclE2OE cells can be isolated and transplanted to compare their efficiency in tumor formation, survival, and proliferation.

We agree that a BSC-specific overexpression of Bcl-2 or transplantation studies are required to make this conclusion. We have addressed this important point by performing tumour experiments expressing Bcl-2 specifically in the bulge (by crossing LSL-Bcl2-IRES-GFP and K19CreER mice). Importantly, we observed the promotion of tumour initiation in Bcl-2<sup>K19OE</sup> mice to a similar extent seen in the Bcl-2<sup>EOE</sup> mice. Thus, these experiments support our statement that Bcl-2 is protecting particularly SC/tumour-initiating cells from cell death. We have included these new data in Fig. EV4A-D.

9. Fig. 5c. The authors quantified the number of aCas3+ cells, are there any differences in cell proliferation or differentiation between the different systems?

We have now included the data analysing proliferation in new Fig. 6D,E in the revised version of our manuscript. Interestingly, our results showed that Bcl-2 expression reduced proliferation in K15ΔNLef1 and suggested a mechanism of apoptosis-induced proliferation. We have detected no major changes in the differentiation of keratinocytes.

10. Fig. 5. The authors should show if there are any differences in apoptosis and cell proliferation in the tumors shown in Fig 5g.

We provide these data in new Appendix Fig. S7E,F of the revised manuscript. Our results show no differences in tumour proliferation and apoptosis between K15ΔNLef1 and K15ΔNLef1/Bcl-2<sup>EOE</sup> mice. These data are in line with our hypothesis that Bcl-2 expressing HFSC are selected to give rise to tumours. Thus, we do not expect differences in proliferation or an increase in apoptosis in tumours of K15ΔNLef1 mice compared to K15ΔNLef1/Bcl-2<sup>EOE</sup> mice.

11. The authors indicate that no changes in tumor type or sebaceous differentiation occur in their model compared to controls. However, in Extended Data Figure 7d, Scd1 expression levels differ from those observed in controls.

Our results from IF stainings for sebaceous markers (Appendix Fig. S7C) show sebaceous gland differentiation and typical morphology of sebaceous tumours (Fig. 7D) for all the tumour samples analysed. Based on the reviewer's comment we have analysed more tumours of both genotypes for SCD1 protein by western blotting and observed variability in tumours of both genotypes. The heterogeneity within the tumour tissue and variation in the cellularity/thickness of the overlying interfollicular epidermis most likely explain the variability seen particularly in the western blot.

Minor points.

1. Page 4. The sentence "a continuous turnover and apoptosis of HG cells" is not clear. Please explain.

We have deleted this sentence from the manuscript to avoid any speculation.

2. Extended data figure 1f. The pictures show a high level of aCas3+ in the HG upon ABT-199 treatment. The representative image differs from the graph, where no significant differences in HG aCas3+ cells are shown.

We observed variations in the number of aCas3<sup>+ve</sup> cells in HGs. The quantification of aCas3<sup>+ve</sup> cells as demonstrated in the graphs presented in Fig. 1B and Fig. EV1D revealed no significant differences in the number of HFs displaying positive HGs following ABT-199 treatment. A possible explanation for aCas3<sup>+ve</sup> cells within the HG following ABT-199 treatment could result from the ABT-199 effect on melanocytes (Nishimura et al. 2011; Nishimura et al. 2002). We have included IF staining for the melanocyte marker Trp2 showing a high number of melanocytes residing in the HG (new Fig. EV1E).

3. K14Cre- Rosa26LSL.Bcl2.IRES.GFP. A better characterization of the model is missing. What is the ratio of Bcl-2 increase compared to controls? Is Bcl-2 evenly expressed in K14 positive cells, or is there any mosaicism present in their system?

Based on the suggestions made by the reviewer, we provide mRNA expression data for Bcl-2<sup>EOE</sup> mice in the revised manuscript. As shown in new Appendix Fig. S4B, Bcl-2 is on average ~17 times higher expressed in Bcl-2<sup>EOE</sup> mice when compared to litter mate controls. Further we include novel data analysing transgene expression (Bcl-2 and GFP<sup>+</sup> cells) in epidermal tail whole mounts and backskin sections. These results reveal robust transgene expression in Keratin 14<sup>+</sup> cells and their progeny in the HF, SG and IFE (new Appendix Fig. S4C-E).

4. K14Cre- Rosa26LSL.Bcl2.IRES.GFP. The authors document an opposite response during catagen to the one observed previously using another K14 Bcl-2 mouse model, which instead led to an accelerated catagen progression (Müller-Röver et al. 1999). How do the authors reconcile the differences?

Several of the main claims and observations made by these authors are in contrast to expected functions of an anti-apoptotic factor. We have the following possible explanations: first, the retracting epithelial strand could have been mistaken for an anagen HF (H&E presented in Figure 5; Müller-Röver et al. 2000). In this case, the phenotype would support our more detailed analysis revealing survival of ORS cells. However, more detailed analysis of HF morphology and marker expression from different stages of the hair cycle would be required for a better understanding of the phenotype presented by Müller-Röver et al. Second, we propose that the different K14Cre line (weaker) used by Müller-Röver et al. and the different keratinocyte populations targeted by us (transgene vs. genetic knock in) could be possible reasons for the different phenotypes.

5. I believe reorganizing the manuscript could benefit the flow of ideas. For example, first, show the RNA seq analyses as a tool to address functional heterogeneity between basal and suprabasal cells. Second, focus on the identified cell death and survival pathways in suprabasal cells. Next, show the differential response of sbBSCs to the Bcl-2 inhibitor, to later focus on the potential mechanisms leading to the control of HF regeneration, etc.

We have rearranged the order of experiments introducing the Bcl-2<sup>EOE</sup> mice and revised the manuscript accordingly.

Dear Dr. Niemann,

Thank you for the submission of your revised manuscript to our editorial offices. We have received the reports from two referees that were asked to re-evaluate your study, you will find below. Unfortunately, the original referee #1 (from the submission to The EMBO Journal) never responded to our invitations to re-assess the study. Nevertheless, looking through your p-b-p-response, we consider his/her concerns as adequately addressed. Referee #1 (referee #4 from the submission to The EMBO Journal) now supports the publication of your study in EMBO reports. In contrast, referee #2 (also referee #2 from the submission to The EMBO Journal) remains critical and is not satisfied by the revisions. We have asked you to comment on the report of referee #2.

Now, looking through all this, considering that referee #1 supports publication (also after cross-commenting) and that two more referees were positive already about the previous submission (to The EMBO Journal), and taking into account your response to the concerns of referee #2, we decided to proceed with the manuscript and invite a final revision. Please address the concerns of referee #2 in a detailed point-by-point-response as suggested in your letter. Moreover, please add the data you mention in your letter (to monitor platelet counts and thrombopoiesis in the mice exposed to ABT-199 under the experimental setup used to exclude a possible inhibition of Bcl-xL), and please further discuss and mention the different scenarios indicated by referee #2 in the final revised manuscript text.

I also have these editorial requests I ask you to address:

- Please call out the single figure panels in a sequential manner (or re-order the figure panels). Presently, Figs. 4F-H is called out after Fig 5, Fig EV5A is called out before Fig EV2 and Fig EV5B is called out before Fig EV4.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV and Appendix figures), and that statistical testing has been done where applicable.

In Figure 6A you show error bars, although you indicate in the legend that only two replicates are shown. Please show the two datasets separated here, without error bars. This is much more transparent and illustrates better the data. Or add a third replicate to do proper statistics.

- Could statistical testing be also performed for the diagrams in Figs. 1B (hair germ), 1D (bBSC), 2D (Runx1), 4C, EV1 (hair germ), EV2E (for those graphs missing testing), EV2A/B (for those graphs missing testing), S1B (Bcl2), S1C (Igr1, Bcl2), S2A, S4B and S7 E/F? If testing has been done, but there is no significant difference, please also indicate this in the diagrams.

- I would suggest moving the table with the primers in the methods section to the Appendix (Appendix Table S1) and add a call-out. Otherwise this needs to become Table 1 (with that callout) and the table needs to be moved with a title and a legend to the end of the manuscript.

- Please combine the funding information with the Acknowledgements. Please make sure that the funding information added into the online submission system is complete and similar to the one in the manuscript (in the Acknowledgements).

- Please name the 'Declaration of interest/conflict' 'Conflict of interest statement'.
- Please update field F18 of the author checklist, indicating the accession code and the link to the GEO database. Make sure that the data is public upon publication of the paper.
- Please format the references and their callouts according to our journal style:  
<http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>
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In addition, I would need from you:

- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Achim Breiling  
Editor  
EMBO Reports

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Referee #1:

I do not have any further comments about the manuscript. The authors conducted a series of new experiments and modified the text to address the major points I raised in its first version. These, together with the additional changes suggested by the other reviewers, improved the manuscript and made it suitable for publication.

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Referee #2:

In this revised manuscript by Geueke et al., originally submitted to EMBO J., the authors propose that a very specific subset of stem cells in skin, i.e. suprabasal bulge stem cells (sbBSCs), are essential for the cyclical regeneration of hair follicles and are uniquely protected from anoikis-induced cell death by Bcl-2. As before, they come to this conclusion based on results from peripheral administration of ABT-199, an established human BCL-2 and BCL-XL inhibitor, as well as knockout or overexpression of Bcl-2 in K14+ cells in mouse skin. The authors have responded to this reviewer's previous criticisms by performing some additional experiments, including confirmation that sbBSCs are in fact Casp3+ following treatment with ABT-199 (which is now much more convincing), as well as staining for GFP and Bcl-2 in sections from Bcl-2 transgenic mice, confirming that Bcl-2 is indeed expressed in all K14+ cells in the skin.

Unfortunately, the over-arching concerns raised previously remain. The authors still have not

provided any "direct" evidence that Bcl-2 is uniquely required for the maintenance of sbBSCs, nor that that anoikis is mediated by Bim and/or Bmf. ABT-199 is still not selective for BCL-2 - yes, it has a bit lower  $K_i$  for BCL-2 than BCL-xL but it is still a nanomolar inhibitor for both proteins and it is very unlikely that, when given to a mouse at the dose chosen, it selectively inhibits only Bcl-2. Moreover, the fact that ABT-199 is given peripherally cannot rule out the possibility that its effects on skin are indirect. The authors emphasize that Bcl-2 mRNA expression is relatively higher in sbBSCs than other BSCs and cell-types in the bulge, but they admit that expression of Bcl-2 at the protein level is undetectable, even though they can readily detect Bcl-2 protein in sbBSCs in their transgenic mice. Can such low, undetectable levels of endogenous Bcl-2 really protect sbBSCs from anoikis? Perhaps, but the authors haven't proven it.

The more direct approach to KO Bcl-2 in K14+ cells is better, but again the KO is not specific to sbBSCs, and in any event, the authors did not observe specific depletion of sbBSCs, even in their K14-restricted Bcl-2 conditional KO, which might be expected if sbBSCs are in fact uniquely dependent upon Bcl-2 for survival, nor was there an accumulation of sbBSCs in their Bcl-2 transgenic mice. The authors argue that perhaps sbBSCs have compensated for the KO of Bcl-2 by upregulating the expression of other antiapoptotic Bcl-2 family members, but they provide no evidence of this. All told, it is equally plausible that indirect effects of ABT-199 on other cell types is responsible for the cell death of sbBSCs, or perhaps it's due to the combined inhibition of Bcl-2 and Bcl-xL in sbBSCs, as previously questioned. To be clear, this reviewer appreciates the difficulty in conducting these genetic experiments, but the authors are trying to make very specific claims about sbBSCs that may be true but are simply not backed up with definitive experiments. Toning down their most important conclusions with words like "may" is unfortunately not a substitution for more definitive experiments.

Finally, as noted before, the fact that overexpression of Bcl-2 in BSCs enhances DMBA-induced skin cancer is not surprising, given that BSCs are known to be the essential targets of DMBA; and the authors have not demonstrated that sbBSCs per se are the specific targets of DMBA or are uniquely responsible for giving rise to tumors.

### Point by point response

First of all, we are glad that reviewer #1 (former referee #4) appreciates the additional work and experimental data addressing the major critical points that improved the manuscript and now finds our revised manuscript suitable for publication.

Here we would like to comment on the report and issues raised by referee #2.

Referee #2:

*"In this revised manuscript by Geueke et al., originally submitted to EMBO J., the authors propose that a very specific subset of stem cells in skin, i.e. suprabasal bulge stem cells (sbBSCs), are essential for the cyclical regeneration of hair follicles and are uniquely protected from anoikis-induced cell death by Bcl-2. As before, they come to this conclusion based on results from peripheral administration of ABT-199, an established human BCL-2 and BCL-XL inhibitor, as well as knockout or overexpression of Bcl-2 in K14+ cells in mouse skin. The authors have responded to this reviewer's previous criticisms by performing some additional experiments, including confirmation that sbBSCs are in fact Casp3+ following treatment with ABT-199 (which is now much more convincing), as well as staining for GFP and Bcl-2 in sections from Bcl-2 transgenic mice, confirming that Bcl-2 is indeed expressed in all K14+ cells in the skin."*

We are pleased that this referee is now convinced by our important observation that sbBSCs are aCasp3 positive and undergo apoptosis following ABT-199 treatment.

*"Unfortunately, the over-arching concerns raised previously remain. The authors still have not provided any "direct" evidence that Bcl-2 is uniquely required for the maintenance of sbBSCs, nor that that anoikis is mediated by Bim and/or Bmf."*

In our revised manuscript we do not make these conclusions. Instead we state on page 6/7: "Alternatively, although established as highly selective Bcl-2 inhibitor (Souers, Levenson et al., 2013), ABT-199 treatment may have interfered with other Bcl-2-family members or other anti-apoptotic proteins expressed by sbBSCs."

Furthermore, and as explained in our point-by-point response to the reviewer #1 (former rev #4) point #3, we do not predicate that "anoikis is mediated by Bim and/or Bmf" in our model. On page 7 of our revised manuscript we state that the RNA-seq and qRT-PCR data analysing sbBSCs (Fig 3B and C) suggest a mechanism of detachment-induced cell death. The discussion of this particular result is purely based on published work (Taddei, Giannoni et al., 2012; Puthalakath, Villunger et al., 2001; Taddei et al., 2012). In this context, we also explained that it is not possible to experimentally assess anoikis during HF regeneration under physiological conditions in vivo.

*"ABT-199 is still not selective for BCL-2 - yes, it has a bit lower  $K_i$  for BCL-2 than BCL-xL but it is still a nanomolar inhibitor for both proteins and it is very unlikely that, when given to a mouse at the dose chosen, it selectively inhibits only Bcl-2."*

In our point-by-point response to this reviewer (point #2) we already referenced the extensive biochemical characterization of ABT-199 by Sours et al (Nature medicine 2013, PMID: 23291630) demonstrating that ABT-199 has a subnanomolar affinity for BCL-2 with a  $K_i < 0.010$  nM (equivalent to 10pM) and bound over three orders of magnitude less avidly to BCL-xL ( $K_i = 48$  nM)". The affinity of ABT-199 to BCL-2 is around 4800 times higher than to BCL-xL!

*"Moreover, the fact that ABT-199 is given peripherally cannot rule out the possibility that its effects on skin are indirect."*

We observe apoptosis of sbBSCs within 12 h following a single ABT-199 treatment using 25mg/kg or 50mg/kg (compared to 100mg/kg ABT-199 used in most of the published in vivo studies, e.g. Vandenberg Blood 2013, PMID: 23341542; Vandenberg Blood 2013,

PMID: 23341542). Moreover, only the cell population expressing the highest level of Bcl-2 in the skin is responding, thus strongly arguing against a non-cell-autonomous effect of the selective inhibitor.

*“The authors emphasize that Bcl-2 mRNA expression is relatively higher in sbBSCs than other BSCs and cell-types in the bulge, but they admit that expression of Bcl-2 at the protein level is undetectable, even though they can readily detect Bcl-2 protein in sbBSCs in their transgenic mice. Can such low, undetectable levels of endogenous Bcl-2 really protect sbBSCs from anoikis? Perhaps, but the authors haven’t proven it.”*

Although it is true, that we did not detect Bcl-2 by IF staining in wildtype mouse skin, we do show Bcl-2 protein expression by western blot in isolated wild-type mouse skin epithelium (Fig. EV 4A). Thus, the statement of ‘undetectable levels of endogenous Bcl-2’ is not correct. Our RNA expression data show a robust Bcl-2 expression and detect strong expression of Bcl-2 in sbBSCs when compared to bBSC and non-Bulge populations (Fig. 2A). We thus conclude that Bcl-2 RNA and protein expression is highest in sbBSCs. This fits well with our observation, that sbBSCs specifically respond to ABT-199 and suggests a protective role of Bcl-2 in sbBSCs.

*“The more direct approach to KO Bcl-2 in K14+ cells is better, but again the KO is not specific to sbBSCs, and in any event, the authors did not observe specific depletion of sbBSCs, even in their K14-restricted Bcl-2 conditional KO, which might be expected if sbBSCs are in fact uniquely dependent upon Bcl-2 for survival, nor was there an accumulation of sbBSCs in their Bcl-2 transgenic mice. The authors argue that perhaps sbBSCs have compensated for the KO of Bcl-2 by upregulating the expression of other antiapoptotic Bcl-2 family members, but they provide no evidence of this.”*

As discussed in our manuscript, the upregulation of other anti-apoptotic factors seems a plausible explanation for the observation. However, the mammalian skin epithelium is a highly plastic tissue and thus we envision that other mechanisms of compensation are also possible. These mechanistic studies of plasticity of different HF stem and progenitor compartments are not the main focus of our study.

The more definitive experiment that this reviewer alluded to regarding specific depletion of sbBSCs is not possible with the current knowledge and tools in the field.

Further, we did not expect an accumulation of sbBSCs in Bcl-2 transgenic mice since, and as shown by our experiments, the expression of Bcl-2 is not instructive to change the ‘type’ of BSC population (Fig 4C).

*“All told, it is equally plausible that indirect effects of ABT-199 on other cell types is responsible for the cell death of sbBSCs, or perhaps it’s due to the combined inhibition of Bcl-2 and Bcl-xL in sbBSCs, as previously questioned.”*

As discussed above, we observe apoptosis of sbBSCs within 12 h following a single ABT-199 treatment using 25mg/kg or 50mg/kg (compared to 100mg/kg ABT-199 used in most of the published in vivo studies, see above). Moreover, only the cell population expressing the highest level of Bcl-2 in the skin is responding, thus arguing against a non-cell-autonomous effect of the selective inhibitor.

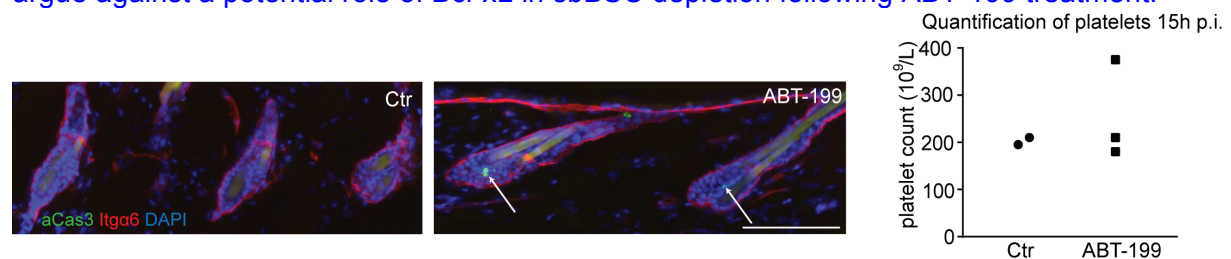
In contrast to BCL-2, antagonising of Bcl-xL using ABT263 (navitoclax) led to thrombocytopenia (Schoenwaelder et al, Blood 2011, PMID: 21673344), which has been explained by platelet survival being dependent on Bcl-xL (Mason et al, Cell 2007, PMID: 17382885). This prompted the development of ABT-199, a modified BH3-mimetic derivative of ABT-263, which maintains specificity for BCL-2, but lacks affinity for BCL-xL (Sours et al, Nature medicine 2013, PMID: 23291630). It is important to note that BCL-2 is dispensable for thrombopoiesis (Debrincat et al Cell Death and Disease 2015, PMID: 25880088) and a number of previous studies already excluded a relevant alteration of platelets in patients and also in mice after exposure to ABT-199 (Vogler et al, Br J Haematol 2013, PMID: 23826785; Ganzel et al, Leukemia and Lymphoma 2020, PMID: 32420775;

Vandenberg Blood 2013, PMID: 23341542; Sours et al, Nature medicine 2013, PMID: 23291630).

In our revised version of the manuscript we also show that in contrast to Bcl-2, Bcl-xL is not differentially expressed in sbBSC (Exp. View Fig. 2A) further arguing against Bcl-xL as a main target in the ABT-199 response.

In addition, we even mentioned the -very unlikely- possibility of potential contribution of other Bcl-2 protein members on page 6 of the revised manuscript stating: "Alternatively, although established as highly selective Bcl-2 inhibitor (Souers, Levenson et al., 2013), ABT-199 treatment may have interfered with other Bcl-2-family members or other anti-apoptotic proteins expressed by sbBSCs."

We do agree that overwhelming amounts of ABT-199 may also interfere with BCL-xL. In order to exclude a possible inhibition of Bcl-xL, we have monitored platelet counts in our mice exposed to ABT-199 under the experimental setup specifically used in our studies (50mg/kg compared to 100mg/kg used in most of the in vivo studies mentioned above and analysis 15 hours following treatment). As demonstrated in the figure below, cell death of sbBSCs (detected by aCas3 IF staining) is detected 15h following ABT-199 administration. However, no changes in number of platelets are observed showing that ABT-199 does not affect Bcl-xL-dependent platelet survival. Thus, together with published results our data argue against a potential role of Bcl-xL in sbBSC depletion following ABT-199 treatment.



**Figure: ABT-199 treatment does not affect platelets.**

Mice were treated with ABT-199 (i.p. 50mg/kg) and analysed 15h later for aCas3 (IF, green) and platelet count. Note specific aCas3 staining of sbBSCs (arrows) and no significant changes in number of platelets.

*"To be clear, this reviewer appreciates the difficulty in conducting these genetic experiments, but the authors are trying to make very specific claims about sbBSCs that may be true but are simply not backed up with definitive experiments. Toning down their most important conclusions with words like "may" is unfortunately not a substitution for more definitive experiments."*

As this reviewer already rightly acknowledged, genetic experiments targeting specifically sbBSCs are not possible using existing knowledge and tools for sbBSCs.

As we explained previously in our point-by-point response to this reviewer (point#3), only the data emanating from the ABT-199 treatment revealed the sbBSC-specific role for anti-apoptotic proteins. The rationale for Bcl-2 overexpression in mouse epidermis was to test if Bcl-2 plays an instructive role and is able to change composition and function of BSCs in vivo and to investigate the consequences of blocking apoptosis for HF/tissue regeneration and not to investigate the role in sbBSCs.

In our revised manuscript, we avoid making claims not supported by the data. Instead we discuss the most likely molecular and cellular mechanisms suggested by the results and we take other possibilities into consideration. Rather it seems that the reviewer is asking to provide proof for claims that are not made by us.

*"Finally, as noted before, the fact that overexpression of Bcl-2 in BSCs enhances DMBA-induced skin cancer is not surprising, given that BSCs are known to be the essential targets of DMBA; and the authors have not demonstrated that sbBSCs per se are the specific targets of DMBA or are uniquely responsible for giving rise to tumors."*

The results of the skin cancer experiments might be not surprising, however, the experiments need to be performed to show the actual outcome and to provide solid evidence. In our

manuscript we neither claim/conclude that sbBSCs are the specific targets of DMBA nor that they are uniquely responsible for giving rise to tumours.

As for the formation of skin tumours, previous work has shown that non-BSCs can also give rise to tumours and the cell-of-origin is still not completely resolved for all the different types of skin tumours. Importantly, our novel data support that BSCs can give rise to sebaceous skin tumours.

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Germany

Dear Dr. Niemann,

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Catherin Niemann  
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### Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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  $n \leq 5$ , 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

#### 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?                                                                                     | N >= 3 biological replicates and experiments were repeated at least two more times                                                                                                                                                                                                                                |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.                                                                       | For tumor experiments sample size (#mice) was chosen based on previous and/or preliminary experiments. For all other experiments sample size was calculated according to needs and statistical relevance                                                                                                          |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?                                                      | Generally all animals with noticeable problems were excluded. Especially skin problems, not related to experimental approach/treatment were excluded from the experiments. I.e. scratches, wounds, from fightings etc. For tumor experiments criteria were also pre-established in the licence for animal testing |
| 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.                | If not stated otherwise, animals from the same sex were used. Usually female mice were used. Animals from same sex were allocated randomly to treatments.                                                                                                                                                         |
| For animal studies, include a statement about randomization even if no randomization was used.                                                                                          | see above                                                                                                                                                                                                                                                                                                         |
| 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. | For most experiments no blinding of the investigator was possible                                                                                                                                                                                                                                                 |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done                                                                                                | no blinding was done                                                                                                                                                                                                                                                                                              |
| 5. For every figure, are statistical tests justified as appropriate?                                                                                                                    | yes                                                                                                                                                                                                                                                                                                               |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.                                                                      | If not stated otherwise data met the assumptions of the normal distribution.                                                                                                                                                                                                                                      |
| Is there an estimate of variation within each group of data?                                                                                                                            | The F test for variances was run together with each statistical test with GraphPad Prism.                                                                                                                                                                                                                         |
| Is the variance similar between the groups that are being statistically compared?                                                                                                       | The variances were not statistically different in most cases. If they were different, the statistical tests were re-run with the Welch option in order to confirm the results of the first test (with GraphPad Prism).                                                                                            |

#### C- Reagents

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<http://www.ebi.ac.uk/ega>  
  
<http://biomodels.net/>  
  
<http://biomodels.net/miriam/>  
<http://iji.biochem.sun.ac.za>  
[http://oba.od.nih.gov/biosecurity/biosecurity\\_documents.html](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 ( <a href="#">see link list at top right</a> ), 1DegreeBio ( <a href="#">see link list at top right</a> ). | K15 (Neomarkers, #MS-1068-P0, Waseem A, et. al. Journal Invest Dermatology, 1999, 112(3):362-9.), K14 ( <a href="https://www.antibodypedia.com/gene/3600/KRT14/antibody/1457740/PRB-155P">https://www.antibodypedia.com/gene/3600/KRT14/antibody/1457740/PRB-155P</a> ), K10 ( <a href="https://www.antibodypedia.com/gene/1606/KRT10/antibody/1457737/PRB-159P">https://www.antibodypedia.com/gene/1606/KRT10/antibody/1457737/PRB-159P</a> ), K6 (Covance, PRB-169P, Kittrell FS, et al. 2011. Breast Cancer Res.13:41.), active Caspase 3 ( <a href="https://www.antibodypedia.com/gene/1222/CASP3/antibody/697828/AF835">https://www.antibodypedia.com/gene/1222/CASP3/antibody/697828/AF835</a> ), BrdU (BD Biosciences, 347580, <a href="https://www.citeab.com/antibodies/2414712-347580-purified-mouse-anti-brdu">https://www.citeab.com/antibodies/2414712-347580-purified-mouse-anti-brdu</a> ), P-Cadherin (Invitrogen, 13-20002, <a href="https://antibodyregistry.org/search.php?q=AB_86574">https://antibodyregistry.org/search.php?q=AB_86574</a> ), Nfatc1 (DSHB, 7A6, <a href="https://antibodyregistry.org/search.php?q=AB_2152505">https://antibodyregistry.org/search.php?q=AB_2152505</a> ), Sox9 ( <a href="https://www.antibodypedia.com/gene/915/SOX9/antibody/552321/AB5535">https://www.antibodypedia.com/gene/915/SOX9/antibody/552321/AB5535</a> ), Scd1 ( <a href="https://www.antibodypedia.com/gene/1335/SCD/antibody/2365999/MAB4404">https://www.antibodypedia.com/gene/1335/SCD/antibody/2365999/MAB4404</a> ), BrdU (Bio-Rad, OBT0030, <a href="https://www.citeab.com/antibodies/122047-obt0030-rat-anti-brdu">https://www.citeab.com/antibodies/122047-obt0030-rat-anti-brdu</a> ), K15 (Progen, GP-CK15), Integrin alpha6 (BD Biosciences, 555734, <a href="https://www.citeab.com/antibodies/2408351-555734-bd-pharmingen-purified-rat-anti-human-cd49f1">https://www.citeab.com/antibodies/2408351-555734-bd-pharmingen-purified-rat-anti-human-cd49f1</a> ), Integrin alpha6-PE (BD Biosciences, 555736, <a href="https://www.citeab.com/antibodies/2410700-555736-bd-pharmingen-pe-rat-anti-human-cd49f1des-f8de48b65d94e249">https://www.citeab.com/antibodies/2410700-555736-bd-pharmingen-pe-rat-anti-human-cd49f1des-f8de48b65d94e249</a> ), gammaH2AX ( <a href="https://www.antibodypedia.com/gene/3220/H2AX/antibody/108086/9718">https://www.antibodypedia.com/gene/3220/H2AX/antibody/108086/9718</a> ), K71, K72, K75, K85 (Progen, 70022.Sun, X. et al. |
|                                                                                                                                                                                                                                                                                                                                                                  | Coordinated hedgehog signaling induces new hair follicles in adult skin. Elife. 9, (2020)), Bcl-2 (BD Bioscience, 610539, <a href="https://www.citeab.com/antibodies/2412426-610539-bd-transduction-laboratories-purified-mouse-des-bd01c319a5af435b">https://www.citeab.com/antibodies/2412426-610539-bd-transduction-laboratories-purified-mouse-des-bd01c319a5af435b</a> ), Trp2 (Santa Cruz, sc-10451, doi: 10.7554/eLife.06857.), GFP-FITC (Rockland, 600-102-215, <a href="https://www.citeab.com/antibodies/1906864-600-102-215-anti-gfp-goat-antibody-fluorescein-con-des-ce26157958fd6b47">https://www.citeab.com/antibodies/1906864-600-102-215-anti-gfp-goat-antibody-fluorescein-con-des-ce26157958fd6b47</a> ), CD34-eFluor660 (eBioscience, 50-0341-82, <a href="https://www.citeab.com/antibodies/2038653-50-0341-cd34-monoclonal-antibody-ram34-eFluor-660-des-68eda64a9fe2b65e">https://www.citeab.com/antibodies/2038653-50-0341-cd34-monoclonal-antibody-ram34-eFluor-660-des-68eda64a9fe2b65e</a> ), GAPDH (Cell Signaling, 5174, <a href="https://www.citeab.com/antibodies/125120-5174-gapdh-d16h11-xp-rabbit-mab7des-b9d55a5a79b917e">https://www.citeab.com/antibodies/125120-5174-gapdh-d16h11-xp-rabbit-mab7des-b9d55a5a79b917e</a> ), alpha-actinin (Cell Signaling, 6487, <a href="https://www.citeab.com/antibodies/125854-6487-actinin-d6f6-xp-rabbit-mab7des-1fb6e0fd7aea3b33">https://www.citeab.com/antibodies/125854-6487-actinin-d6f6-xp-rabbit-mab7des-1fb6e0fd7aea3b33</a> )                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| 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.                                                                                                                                                                                                                                                            | mouse, C57Bl/6 background, f/m, 3-20 weeks, K14Cre, K19CreER, Lgr5CreER-eGFP, Lrig1CreER-eGFP, K15 <sup>fl</sup> INLef1, Bcl-2lox and Rosa26LSL.IRES.GFP. Mice were group-housed on a 12:12 hours day (light) and night (dark) cycle in specific pathogen free (SPF) cages |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.                                                                                                                                                                                                                                                                       | in vivo experiments were carried out according to ethical guidelines and the animal license given by the State Office North-Rhine Westfalia                                                                                                                                |
| 10. We recommend consulting the ARRIVE guidelines ( <a href="#">see link list at top right</a> ) (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 ( <a href="#">see link list at top right</a> ) and MRC ( <a href="#">see link list at top right</a> ) recommendations. Please confirm compliance. | we confirm compliance to these guidelines.                                                                                                                                                                                                                                 |

#### 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 ( <a href="#">see link list at top right</a> ) and submit the CONSORT checklist ( <a href="#">see link list at top right</a> ) 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 ( <a href="#">see link list at top right</a> ). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.                                                                                          | NA |

#### F- Data Accessibility

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                   |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions                                                                                                                                             | RNA sequencing data deposited on GEO database under the name of "RNA sequencing of isolated murine basal and suprabasal hair follicle bulge stem cells" with accession: GSE166921 |
| 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 ( <a href="#">see link list at top right</a> ) or Figshare ( <a href="#">see link list at top right</a> ).                                                                                                                                                                                                                                             | see above                                                                                                                                                                         |
| 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 ( <a href="#">see link list at top right</a> ) or EGA ( <a href="#">see link list at top right</a> ).                                                                                                                                                                                                                                      | NA                                                                                                                                                                                |
| 21. 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 ( <a href="#">see link list at top right</a> ) and deposit their model in a public database such as Biomodels ( <a href="#">see link list at top right</a> ) or JWS Online ( <a href="#">see link list at top right</a> ). 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

|                                                                                                                                                                                                                                                                                                                                   |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents ( <a href="#">see link list at top right</a> ) and list of select agents and toxins (APHIS/CDC) ( <a href="#">see link list at top right</a> ). According to our biosecurity guidelines, provide a statement only if it could. | NA |
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