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Glutamate transporter Slc1a3 mediates inter-niche stem cell activation during skin growth

Bettina Reichenbach, Johanna Classon, Tomomi Aida, Kohichi Tanaka, Maria Genander and Christian Göritz

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Transaction Report:

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

1st Editorial Decision 19th October 2017

Thank you for the submission of your manuscript (EMBOJ-2017-98280) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #2 points out that some conclusions are not sufficiently well supported by the current data and thus states the need for you to corroborate your findings on a role of Slc1a3 in the interfollicular epidermis and provide genetic proof for involvement of glutamate transport. Referees #1 and #3 agree in that more molecular insights into co-dependence of Slc1a3 and mGluR5 would be required to support the model proposed. In addition, referees #1 and #3 list a number of technical issues and controls regarding proliferation/hair regrowth assays and statistical analyses applied, that need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I agree with the referees that the manuscript would strongly benefit from more refined characterization of glutamate signaling involved in the inter niche coordination.

REFEREE REPORTS

Referee #1:

The manuscript describes the glutamate transporter Slc1a3 as a novel, transiently expressed marker of activated stem cells in the hair follicle, sebaceous gland and interfollicular epidermis compartments of the mouse epidermis, and identifies a role for this transporter in coordinating

proliferation between these compartments.
This is an exciting manuscript with high quality experimental data. The findings are novel as very few markers for activated stem cells exist. In addition, the manuscript identifies a novel mechanisms of intercompartment crosstalk. The manuscript will be of interest to the readership of EMBO Journal. I have only minor comments

1. The manuscript describes a wide range of findings and presents a large number of experimental data, but in its current condensed form it is somewhat difficult to follow. In addition, large number of key data is placed in supplementary figures. I recommend extending the format into a full manuscript form with a proper introduction and discussion, as well as moving data from the supplementary figures into main figures.

2. Reduced fur density (number of hair shafts/follicle) is used as a readout for reduced HFSC activation and proliferation (Fig. 1n, Extended Data Fig. 2l). This is a very indirect readout as numerous other pathological situations (see pili multigemini) could cause a similar phenotype. The authors should use depilation-based anagen induction and quantitative hair regrowth as an assay to address the loss of function of Slc1a3

3. Showing that Slc1a3-expressing cells have higher colony-forming ability (or that they form bigger colonies) would strengthen the conclusion that the effect of SLc1a3 on stem cell activation is cell autonomous

Referee #2:

IFE, it is somehow disappointing to see that the expression of Slc1a3 (Cre) is restricted to such a small fraction of proliferating cells (<20%) not varying between anagen and telogen. In general this is further reflected by the fact that Slc1a3-Cre needs to be injected 5 consecutive days with tamoxifen. This might argue against authors claim that Slc1a3 is an important contributor of all epidermal compartments. The rasless experiment only goes on to show that upon permanent removal of ras these compartments do not proliferate. Given the fact that Slc1a3 expressing cells are widespread in anagen and occupy a large part of the epidermis in lineage tracing over time such finding is not surprising.
Finally the assumption that glutamate transport is linked with glutamate receptor 5 signalling is supported by pharmacological inhibition and would be best served by the genetic model available to authors. If slc1a3-/- mice show no response to Glutamate receptor inhibitor or to MTEP this will be additional proof of the appropriate targeting of these drugs.

Overall, authors convincingly report the activation of Slc1a3 in all three compartments in stem/progenitor populations. The fate of these cells should be however clarified especially in the hair follicle. Slc1a3 functions in epidermal cells remains in my opinion inconclusive and must be studied further to convincingly show a cell-intrinsic activity possibly through the glutamate receptor 5.

Referee #3:

Using murine skin as the model, Classon et al. address an important question on the coordination of tissue growth when it is driven by multiple stem cell niches. Murine skin encompasses at least three different niches for epithelial stem cells: the hair follicle (HF), the sebaceous gland (SG), and the interfollicular epidermis (IFE) that all replenish the tissue (under homeostasis each contributing to their own sub-compartment) but with different dynamics: the first renews cyclically while the latter two continuously replace lost cells. Significantly, the manuscript reports that hair growth is accompanied by SG and IFE expansion, and identifies glutamate transporter Slc1a3 as an effector of this coordination. Fate mapping analysis indicates Slc1a3 is transiently expressed in all three compartments, and gene deletion experiments suggest that loss of Slc1a3 uncouples SG and IFE expansion from the hair cycle. Modulation of glutamate receptor 5 (MGluR5) activity by topical drugs mimics the effects of Slc1a3 deletion or inhibition.

The manuscript has been written in a concise way (apparently initially for another journal) and would greatly benefit and become more reader-friendly if expanded to the EMBO J format: a more thorough Introduction, and in particular in depth Discussion where the findings are contrasted to other stem cell systems will be highly useful. This will also make the paper more appealing to a non-specialized audience. In its current form, it is at risk of losing a broader readership.

The manuscript is thorough, largely well written, and the data are convincing. A few issues should be addressed:

1.
All data with error bars are reported as mean +- standard error of the mean. How do the authors justify the use of SEM? In my opinion, the reader should be aware of the variation in the data points and data should be reported as mean +- standard deviation.

2.
Although the number of mice analyzed is reported in Materials and Methods, and it also includes the number of items analyzed, the latter is often vaguely reported: e.g. "15-20 vertically sectioned HFs..." (line 260), "up to 20 SG sections per animal" (line 272), "up to 20 basal cell clusters were analyzed" (line 281) etc. For these reasons, the reader cannot deduce the actual sample size used for statistical analyses. The exact sample sizes should be reported.

3.
I was a bit confused about the results on the hair density in aged control and Slc1a3-/- mice. It is reported that the number of HFs remains the same compared to controls even in old animals (Expanded data Fig. 2k) but that the percentage of HFs with more than 2 hair shafts is greatly reduced and hence the fur is less dense. Does this in fact mean that Slc1a3 is needed for anchoring the hair shaft? Can that be excluded? Or are the authors proposing that the number of hair cycles that mutant mice undergo is about half of that than in controls?

4.
When comparing the anagen and telogen regions within each aged animal, the authors write "we found increased proliferation of SG and IFE basal cells in Slc1a3+/+ mice during anagen, in line with our data from young mice." (lines 70-71). However, the p-value for these data are 0.09 (Fig. 1r). Or did I misunderstand these data?

5.
Please explain in more detail what exactly is shown in Figure 3a - this is challenging to follow, but would be important as it forms the basis for following experiments.

1st Revision - authors' response 26th January 2018

Referee #1:

The manuscript describes the glutamate transporter Slc1a3 as a novel, transiently expressed marker of activated stem cells in the hair follicle, sebaceous gland and interfollicular epidermis compartments of the mouse epidermis, and identifies a role for this transporter in coordinating proliferation between these compartments. This is an exciting manuscript with high quality experimental data. The findings are novel as very few markers for activated stem cells exist. In addition, the manuscript identifies a novel mechanism of intercompartment crosstalk. The manuscript will be of interest to the readership of EMBO Journal. I have only minor comments.

We were happy to read that the reviewer found our study exciting and of high quality. We are grateful for the comments as they helped us to clarify the raised points, which improved the manuscript.

1. The manuscript describes a wide range of findings and presents a large number of experimental data, but in its current condensed form it is somewhat difficult to follow. In addition, large number of key data is placed in supplementary figures. I recommend extending the format into a full

manuscript form with a proper introduction and discussion, as well as moving data from the supplementary figures into main figures.

We thank the reviewer for this comment. We have extended the manuscript to a full article format including introduction and discussion. We have extended the number of main figures from 4 to 7, which now contain all key data, as well as 5 expanded view figures.

2. Reduced fur density (number of hair shafts/follicle) is used as a readout for reduced HFSC activation and proliferation (Fig. 1n, Extended Data Fig. 2l). This is a very indirect readout as numerous other pathological situations (see pili multigemini) could cause a similar phenotype. The authors should use depilation-based anagen induction and quantitative hair regrowth as an assay to address the loss of function f Slc1a3.

We thank the reviewer for this relevant question.
We have now added more data from *Slc1a3*$^{-/-}$ mice at 3 months and 1,5-2 years of age to further clarify this point.
From the 3rd anagen phase on, hair cycle phases are no longer synchronized. Instead, anagen is randomly initiated at different sites. We found that at 3 months of age in average 32,5±7,2% of the back-skin area of *Slc1a3*$^{+/+}$ mice was in 3rd anagen, compared to 6,6±4,9% in *Slc1a3*$^{-/-}$ animals, measured by the area of pigmented back-skin characteristic for anagen follicles (Fig 2H and EV2D). Furthermore, *Slc1a3*$^{+/+}$, but not *Slc1a3*$^{-/-}$, hair follicles contained one pigmented anagen hair shaft consistent with them having entered growth phase (Fig 2I), indicating that *Slc1a3*$^{-/-}$ mice fail to efficiently initiate anagen.
Quantification of club hairs at 3 months of age revealed no statistically significant difference between *Slc1a3*$^{+/+}$ and *Slc1a3*$^{-/-}$ mice (Fig EV2E), suggesting that club hairs are not selectively lost in *Slc1a3*$^{-/-}$ hair follicles. However, the number of hair follicles with three club hairs tends to be reduced in *Slc1a3*$^{-/-}$ compared to *Slc1a3*$^{+/+}$ animals (Figure EV2E). This minor reduction of club hairs at 3 months is mirrored by the significant reduction of club hairs seen in aged *Slc1a3* deficient mice (Figure 2K and 2L).
The deficiency in anagen initiation was also pronounced in aged (1.5-2 years old) animals. Aged *Slc1a3*$^{+/+}$ mice presented with more pigmented spots than aged *Slc1a3*$^{-/-}$ mice, suggesting that in absence of *Slc1a3* normal anagen initiation is disturbed (Fig 2J).

To exclude loss of hairs due to adhesion defects in *Slc1a3*$^{-/-}$ mice we plucked hairs and analyzed to what extent cells and which cell type were attached to the plucked hair shaft. When comparing *Slc1a*$^{+/-}$ and *Slc1a3*$^{-/-}$ animals, we did not find any differences in the number of K6^{+} (inner bulge) cells attached to the hair shaft. Furthermore, neither the *Slc1a3*$^{-/-}$ nor the *Slc1a3*$^{+/-}$ had any CD34^{+} outer bulge cells attached to the shafts, suggesting that hair anchoring is not altered in *Slc1a3*$^{-/-}$ animals. This experiment rules out hair shaft loss due to reduced adhesion in the absence of *Slc1a3*. These data are now included in Figure EV2G.

We have included the new data in the Figures 2I, J, EV2E and G and in the text as following:

"From the 3rd anagen phase on, hair cycle phases are no longer synchronized. Instead, anagen is randomly initiated at different sites. We found that at 3 months of age in average 32,5±7,2% of the back-skin area of *Slc1a3*$^{+/+}$ mice was in 3rd anagen, compared to 6,6±4,9% in *Slc1a3*$^{-/-}$ animals, measured by the area of pigmented back-skin characteristic for anagen follicles (Fig 2H and EV2D). The number of club hairs per hair follicle cluster was similar between *Slc1a3*$^{+/+}$ and *Slc1a3*$^{-/-}$ mice (Fig EV2E) suggesting that club hairs are not selectively lost in *Slc1a3*$^{-/-}$ hair follicles. Furthermore, *Slc1a3*$^{+/+}$, but not *Slc1a3*$^{-/-}$, hair follicles contained one pigmented anagen hair shaft consistent with them having entered growth phase (Fig 2I), indicating that *Slc1a3*$^{-/-}$ mice fail to efficiently initiate anagen.
The deficiency in anagen initiation was also pronounced in aged (1.5-2 years old) animals. Aged *Slc1a3*$^{+/+}$ mice presented with more pigmented spots than aged *Slc1a3*$^{-/-}$ mice, suggesting that in absence of *Slc1a3* normal anagen initiation is disturbed (Fig 2J). Although the number of hair follicles was maintained (Fig EV2F) and hair anchoring was not altered in *Slc1a3*$^{-/-}$ mice (Fig EV2G) abrogating *Slc1a3* long-term resulted in reduced fur density. Whereas more than 45% of *Slc1a3*$^{+/+}$ hair follicles contained 3-4 hair shafts, less than 10% of *Slc1a3*$^{-/-}$ hair follicles consisted of groups of more than 2 hair shafts (Fig 2K and L). Together, these data suggest that genetic ablation

of *Slc1a3* leads to reduced hair follicle stem cell activation and proliferation, consequently resulting in disturbed anagen initiation, impaired hair follicle cycling and, over time, reduced fur density."

As suggested by the reviewer, we also performed depilation in adult *Slc1a3*$^{+/-}$ and *Slc1a3*$^{-/-}$ animals to synchronize anagen onset and compare the anagen initiation and progression rate. Five days after depilation, there was no significant difference in the anagen staging comparing *Slc1a*$^{+/-}$ and *Slc1a3*$^{-/-}$ mice (Figure 5O). However, surprisingly we only detected Slc1a3-CreER^{T2}-expressing cells in 12% of anagen I and 13% of anagen II depilation-induced hair follicles compared to 95% and 95% in non-induced first anagen hair follicles, respectively (Figure 5M). Interestingly, at anagen III, when hair follicles regain normal morphology, we found Slc1a3-CreER^{T2} expression at comparable levels in depilation-induced and first anagen hair follicles (Figure 5M and N). Based on this experiment, we suggest that 1) depilation is not a suitable model to assess the role of Slc1a3 in anagen progression, and 2) interestingly, Slc1a3 is not required for the extensive proliferation induced by acute injury such as depilation.

To this end, we also isolated epidermal progenitor cells (using Sca1+ and Itga6+) and hair follicle stem cells (using Sca1-, CD34+ and Itga6+) from wild type mice in anagen. As expected, both epidermal progenitor cells and hair follicle stem cells expressed Slc1a3 mRNA. However, after culturing both cell populations down regulated Slc1a3 expression, indicating that proliferation induced by wounding conditions such as depilation or in vitro culture is Slc1a3 independent. These results are now included in Figure 5P and Q.

3. Showing that Slc1a3-expressing cells have higher colony-forming ability (or that they form bigger colonies) would strengthen the conclusion that the effect of SLc1a3 on stem cell activation is cell autonomous

Since Slc1a3 expression is lost in culture, we could not perform colony-forming assays as suggested by the reviewer. Instead, we re-expressed human Slc1a3 in cultured primary mouse keratinocytes to determine cell autonomous effects on cell proliferation. Early passage keratinocytes were infected with a lentiviral construct containing hSlc1a3 cDNA under a doxycycline inducible promoter. Three days of doxycycline administration resulted in high expression of hSlc1a3 (Figure 5R), and reduced proliferation, as judged by quantitative immunofluorescence using the mitosis marker pH3 (Figure 5S). Hence, re-establishing Slc1a3 in highly proliferative cultured keratinocytes reduces their proliferation rate and supports the notion that Slc1a3 is a cell autonomous modulator of epidermal stem cell activation during homeostasis, but not in wounding.

We have included these data as a new paragraph in the manuscript:

"Injury-induced stem cell activation is Slc1a3 independent
Depilation is considered as a mild injury, leading to hair follicle stem cell proliferation and hair growth. We investigated Slc1a3-CreER^{T2} expression after depilation-induced injury and discovered that in contrast to growth-induced stem cell activation, hair follicle stem cells do not upregulate Slc1a3 after injury. After depilation, hair follicle stem cells in anagen I and II show significantly reduced CreER^{T2} expression when compared to non-induced anagen hair follicles. Interestingly, during anagen III-IV a normal morphology and proliferation pattern is reestablished and CreER^{T2} is expressed in the ORS of depilated and non-induced anagen hair follicles alike (Fig 5M and N). To address the functional role of Slc1a3 in depilation-induced anagen, we analyzed *Slc1a3*$^{+/-}$ and *Slc1a3*$^{-/-}$ hair follicles 5 days after depilation. In contrast to non-induced anagen (Fig 2A-G), the absence of *Slc1a3* did not affect the growth of the depilation-induced new hair follicle (Fig 5O). To address the potential loss of Slc1a3 in a different injury model, we sorted CD34^{+} anagen hair follicle stem cells as well as IFE basal cells (Sca1^{+}/Itga6^{+}) and cultured them under proliferative conditions. While we consistently detected Slc1a3 mRNA in *in vivo* isolated cells, Slc1a3 expression was downregulated in culture (Fig 5P and Q). Re-expression of Slc1a3 in cultured primary mouse keratinocytes (Fig 5R) resulted in reduced proliferation (Fig 5S). These results suggest Slc1a3 as a cell autonomous modulator of epidermal stem cell activation during growth and homeostasis, but not after wounding."

Referee #2:

IFE, it is somehow disappointing to see that the expression of Slc1a3 (Cre) is restricted to such a small fraction of proliferating cells (<20%) not varying between anagen and telogen.

We apologize for not making this clear to the reader. Slc1a3 ($CreER^{T2}$) is transiently expressed in activated basal cells (Figure 5), meaning that at a given time point $CreER^{T2}$ will only be detected in a subset of the cells that have the potential to express Slc1a3 ($CreER^{T2}$). This transient nature of Slc1a3 expression was included in the first manuscript and is now shown in current Figure 5A-C (SG) and 5E-G (IFE). Data included in the first manuscript (now Figure 1F-N) further show that $CreER^{T2}$-expression in the IFE, SG and hair follicle stem cells do in fact significantly increase between telogen and anagen, correlating to the overall increase in proliferation in all three compartments.

In general this is further reflected by the fact that Slc1a3-Cre needs to be injected 5 consecutive days with tamoxifen. This might argue against authors claim that Slc1a3 is an important contributor of all epidermal compartments.

Due to the transient nature of Slc1a3 expression (Figure 5), we decided to inject tamoxifen for 5 consecutive days in order to target a fair number of cells. However, to address the reviewers concern, we reduced tamoxifen administration to two days and compared the number of YFP^+ cells in either the SG or IFE. We found that 2 days (P54 and P55) of tamoxifen administration labels 49% of SGs, compared to 5 days (P51-P55) of tamoxifen, which labels 84% of SGs when analyzed at P68. Similarly, recombination rates increased from 16 to 40 YFP^+ clusters per 10mm of IFE in the same experiment. Hence, extending tamoxifen administration increases, as expected, the number of cells targeted, but does not in any way alter the conclusion of the lineage tracing. In fact, we argue that the broad importance of Slc1a3-expression for the epidermis is revealed not by the exact number of cells labelled, but by the long-term contribution of the labelled stem cells to the IFE (Figure 3J-L).

We have included the new data as following in the text:

"In SGs a small fraction of basal cells expressed Slc1a3 and $CreER^{T2}$ at all times (Fig 1I, J, EV1D, E and EV3E) while a large proportion of lineage traced SGs lost YFP expression over time (Fig 3F and EV3D), suggesting that Slc1a3 expression is transient. To address this question, we reduced tamoxifen administration to two days (P54-55) instead of five (P51-55) (Fig 3E-I) and compared the YFP expression. When analyzed at P68 we detected a significant reduction in the percentage of YFP^+ SGs from 83±3% to 49±12%. Also, the number of YFP^+ clusters/10mm of IFE reduced from 40±5 to 16±6 (Fig EV4A, B). These results suggest that Slc1a3 expression in SG and IFE is changed within days and verifies the 5-day tamoxifen regime."

The rasless experiment only goes on to show that upon permanent removal of ras these compartments do not proliferate. Given the fact that Slc1a3 expressing cells are widespread in anagen and occupy a large part of the epidermis in lineage tracing over time such finding is not surprising.

We again apologize for not making this experiment entirely clear.
Slc1a3 ($CreER^{T2}$) is transiently expressed in hair follicle, SG and IFE stem/progenitor cells (Figure 4 and 5), meaning that at a given time point $CreER^{T2}$ will only be detected in a subset of the cells that have the potential to express Slc1a3 ($CreER^{T2}$). Tamoxifen-dependent, cell-specific deletion of all ras genes in Slc1a3 expressing cells, leads to cell specific impairment of cell division. The impairment will only affect cells, which express Slc1a3 ($CreER^{T2}$) during the time in which tamoxifen is present. The experiment validates the transient expression of Slc1a3 ($CreER^{T2}$) as the impairment of proliferation exceeded the number of Slc1a3 ($CreER^{T2}$) expressing cells at any particular time point. Furthermore, it also showed the potential of the Slc1a3 expressing cells.

Finally the assumption that glutamate transport is linked with glutamate receptor 5 signalling is supported by pharmacological inhibition and would be best served by the genetic model available to authors. If slc1a3-/- mice show no response to Glutamate receptor inhibitor or to MTEP this will be

additional proof of the appropriate targeting of these drugs.

We thank the reviewer for this suggestion. We have treated *Slc1a3*$^{-/-}$ mice with the glutamate receptor inhibitor MTEP and investigated hair cycle progression and SG and IFE growth. While *Slc1a3*$^{-/-}$ as well as MTEP treatment delayed the hair cycle progression, and SG and IFE growth, we did not detect a significant effect of MTEP treatment on *Slc1a3*$^{-/-}$ mice, demonstrating the specificity of the Slc1a3/mGluR5 cooperation. We have included these new data in Figure EV5A and C-F and described them in the text as following:

"Combined application of MTEP and DL-TBOA did not exceed the effect of single compounds, suggesting that Slc1a3 and mGluR5 act on the same pathway (Fig 7C). Furthermore, treatment of *Slc1a3*$^{-/-}$ mice with MTEP had no effect on anagen progression beyond the *Slc1a3*$^{-/-}$ phenotype alone (Fig EV5A), further strengthening the conclusion of Slc1a3 and mGluR5 cooperation. Treatment with L-glutamate only slightly expedited anagen entry compared to vehicle treated animals (Fig EV5B). Moreover, we found that blocking mGluR5 or Slc1a3 significantly reduced anagen-induced growth of SG and IFE. Again, there was no additive effect of the combined antagonist treatment (Fig 7D-G) and no effect of MTEP on *Slc1a3*$^{-/-}$ mice (Fig EV5C-F)."

Overall, authors convincingly report the activation of Slc1a3 in all three compartments in stem/progenitor populations. The fate of these cells should be however clarified especially in the hair follicle.

We are happy to see that the reviewer finds our data regarding Slc1a3 activation convincing.

With regards to the fate of Slc1a3-expressing cells, we have extensive lineage tracing data included in the manuscript for all three compartments discussed. This data has now been moved from supplementary to main figures to make them more visible (Figure 3 and Figure 4). In Figure 3 we follow the fate of Slc1a3-expressing cells targeted in the ORS at P25/P26. Lineage tracing showed that targeted cells in the ORS survive catagen and end up in the new hair germ and bulge in 2nd telogen at P68. We also demonstrate the long-term ability of these cells to act as stem cells by tracing them yet another hair cycle to 3rd telogen (P117) where targeted YFP^{+} cells labelled in the ORS at P25/P26 are again found in both hair germ and the bulge.

In Figure 4 we fate map Slc1a3 expressing cells at two different time points during anagen and determine the fate of these cells and their contribution to the full grown anagen follicle, the following telogen follicle as well as the next following telogen follicle. We clearly describe that Slc1a3^{+} cells contribute to matrix and inner layers of the anagen hair follicle, to CD34^{+} bulge stem cells and K6^{+} inner layer cells in the following telogen.

Figure 3 also contains data describing labelling of Slc1a3-expressing basal cells in either SG or IFE. Recombination is induced at P51-P55 and labelled clones consisting of both basal progenitor cells and supra basal differentiated cells in the IFE can be detected for up to 8 months, demonstrating their stem cell potential (Figure 3J-L). Similarly, recombination of basal Slc1a3-expressing cells in the SG results in the appearance of fully recombined SGs containing both undifferentiated, K5^{+} progenitor cells as well as differentiated FABP^{+} sebocytes for up to 8 months (Figure 3E-I).

Slc1a3 function in epidermal cells remains in my opinion inconclusive and must be studied further to convincingly show a cell-intrinsic activity possibly through the glutamate receptor 5.

In the revised version of the manuscript, we show that culturing of epidermal keratinocytes or hair follicle stem cells leads to downregulation of Slc1a3 (Figure 5P and Q), most likely as a consequence of the wounding induced response cells undergo when put in culture. To demonstrate cell autonomous activity of Slc1a3, we re-expressed human Slc1a3 in cultured primary mouse keratinocytes. Early passage keratinocytes were infected with a lentiviral construct containing hSlc1a3 cDNA under a doxycycline inducible promoter. Three days of doxycycline administration resulted in high expression of hSlc1a3 (Figure 5R), and reduced proliferation, as judged by quantitative immunofluorescence using the mitosis marker pH3 (Figure 5S). Hence, re-establishing Slc1a3 in highly proliferative cultured keratinocytes reduces their proliferation rate and supports the notion that Slc1a3 is a cell autonomous modulator of epidermal stem cell activation during growth and homeostasis, but not after wounding.

Referee #3:

Using murine skin as the model, Classon et al. address an important question on the coordination of tissue growth when it is driven by multiple stem cell niches. Murine skin encompasses at least three different niches for epithelial stem cells: the hair follicle (HF), the sebaceous gland (SG), and the interfollicular epidermis (IFE) that all replenish the tissue (under homeostasis each contributing to their own sub-compartment) but with different dynamics: the first renews cyclically while the latter two continuously replace lost cells. Significantly, the manuscript reports that hair growth is accompanied by SG and IFE expansion, and identifies glutamate transporter Slc1a3 as an effector of this coordination. Fate mapping analysis indicates Slc1a3 is transiently expressed in all three compartments, and gene deletion experiments suggest that loss of Slc1a3 uncouples SG and IFE expansion from the hair cycle. Modulation of glutamate receptor 5 (MGluR5) activity by topical drugs mimics the effects of Slc1a3 deletion or inhibition.

The manuscript has been written in a concise way (apparently initially for another journal) and would greatly benefit and become more reader-friendly if expanded to the EMBO J format: a more thorough Introduction, and in particular in depth. Discussion where the findings are contrasted to other stem cell systems will be highly useful. This will also make the paper more appealing to a non-specialized audience. In its current form, it is at risk of losing a broader readership.

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1. All data with error bars are reported as mean +- standard error of the mean. How do the authors justify the use of SEM? In my opinion, the reader should be aware of the variation in the data points and data should be reported as mean +- standard deviation.

To resolve this question, we have turned to the Author Guidelines regarding statistics published on the EMBO Journal webpage and the article they provide for statistical guidance (Common Statistical Pitfalls in Basic Science, Lisa M Sullivan et al, 2016). The author´s state the following regarding the use of standard deviation and standard error:

“In basic science studies, investigators often move immediately into comparisons among groups. If the outcome being compared among groups is continuous, then means and standard errors should be presented for each group. There is often confusion about when to present the standard deviation or the standard error. Standard deviations describe variability in a measure among experimental units (eg, among participants in a clinical sample), whereas standard errors represent variability in estimates (eg, means or proportions estimated for each comparison group). When summarizing continuous outcomes in each comparison group, means and standard errors should be used.”

Based on Sullivan et al., we display data in the format of means and standard error of the mean. Source data for all graphs are provided.

2. Although the number of mice analyzed is reported in Materials and Methods, and it also includes the number of items analyzed, the latter is often vaguely reported: e.g. "15-20 vertically sectioned HFs..." (line 260), "up to 20 SG sections per animal" (line 272), "up to 20 basal cell clusters were analyzed" (line 281) etc. For these reasons, the reader cannot deduce the actual sample size used for statistical analyses. The exact sample sizes should be reported.

We have now added the exact samples sizes, provided in the figure legends.

3. I was a bit confused about the results on the hair density in aged control and Slc1a3-/- mice. It is reported that the number of HFs remains the same compared to controls even in old animals (Expanded data Fig. 2k) but that the percentage of HFs with more than 2 hair shafts is greatly

reduced and hence the fur is less dense. Does this in fact mean that Slc1a3 is needed for anchoring the hair shaft? Can that be excluded? Or are the authors proposing that the number of hair cycles that mutant mice undergo is about half of that than in controls?

We thank the reviewer for this relevant question.
We have now added additional data from *Slc1a3*$^{-/-}$ mice at 3 months and 1,5-2 years of age to further clarify this point.
From the 3rd anagen phase on, hair cycle phases are no longer synchronized. Instead, anagen is randomly initiated at different sites. We found that at 3 months of age in average 32,5±7,2% of the back-skin area of *Slc1a3*$^{+/+}$ mice was in 3rd anagen, compared to 6,6±4,9% in *Slc1a3*$^{-/-}$ animals, measured by the area of pigmented back-skin characteristic for anagen follicles (Fig 2H and EV2D). Furthermore, *Slc1a3*$^{+/+}$, but not *Slc1a3*$^{-/-}$, hair follicles contained one pigmented anagen hair shaft consistent with them having entered growth phase (Fig 2I), indicating that *Slc1a3*$^{-/-}$ mice fail to efficiently initiate anagen.
Quantification of club hairs at 3 months of age revealed no statistically significant difference between *Slc1a3*$^{+/+}$ and *Slc1a3*$^{-/-}$ mice (Fig EV2E), suggesting that club hairs are not selectively lost in *Slc1a3*$^{-/-}$ hair follicles. However, the number of hair follicles with three club hairs tend to be reduced in *Slc1a3*$^{-/-}$ compared to *Slc1a3*$^{+/+}$ animals (Figure EV2E). This minor reduction of club hairs at 3 months is mirrored by the significant reduction of club hairs seen in aged *Slc1a3* deficient mice (Figure 2K and 2L).
The deficiency in anagen initiation was also pronounced in aged (1.5-2 years old) animals. Aged *Slc1a3*$^{+/+}$ mice presented with more pigmented spots than aged *Slc1a3*$^{-/-}$ mice, suggesting that in absence of *Slc1a3* normal anagen initiation is disturbed (Fig 2J).

To exclude loss of hairs due to adhesion defects in *Slc1a3*$^{-/-}$ mice we plucked hairs and analyzed to what extent cells and which cell type were attached to the plucked hair shaft. When comparing *Slc1a*$^{+/-}$ and *Slc1a3*$^{-/-}$ animals, we did not find any differences in the number of K6^{+} (inner bulge) cells attached to the hair shaft. Furthermore, neither the *Slc1a3*$^{-/-}$ nor the *Slc1a3*$^{+/-}$ had any CD34^{+} outer bulge cells attached to the shafts, suggesting that hair anchoring is not altered in *Slc1a3*$^{-/-}$ animals. This experiment rules out hair shaft loss due to reduced adhesion in the absence of *Slc1a3*. These data are now included in Figure EV2G.

We have included the new data in the Figures 2I, J, EV2E and G and in the text as following:

"From the 3rd anagen phase on, hair cycle phases are no longer synchronized. Instead, anagen is randomly initiated at different sites. We found that at 3 months of age in average 32,5±7,2% of the back-skin area of *Slc1a3*$^{+/+}$ mice was in 3rd anagen, compared to 6,6±4,9% in *Slc1a3*$^{-/-}$ animals, measured by the area of pigmented back-skin characteristic for anagen follicles (Fig 2H and EV2D). The number of club hairs per hair follicle cluster was similar between *Slc1a3*$^{+/+}$ and *Slc1a3*$^{-/-}$ mice (Fig EV2E) suggesting that club hairs are not selectively lost in *Slc1a3*$^{-/-}$ hair follicles. Furthermore, *Slc1a3*$^{+/+}$, but not *Slc1a3*$^{-/-}$, hair follicles contained one pigmented anagen hair shaft consistent with them having entered growth phase (Fig 2I), indicating that *Slc1a3*$^{-/-}$ mice fail to efficiently initiate anagen.
The deficiency in anagen initiation was also pronounced in aged (1.5-2 years old) animals. Aged *Slc1a3*$^{+/+}$ mice presented with more pigmented spots than aged *Slc1a3*$^{-/-}$ mice, suggesting that in absence of *Slc1a3* normal anagen initiation is disturbed (Fig 2J). Although the number of hair follicles was maintained (Fig EV2F) and hair anchoring was not altered in *Slc1a3*$^{-/-}$ mice (Fig EV2G) abrogating *Slc1a3* long-term resulted in reduced fur density. Whereas more than 45% of *Slc1a3*$^{+/+}$ hair follicles contained 3-4 hair shafts, less than 10% of *Slc1a3*$^{-/-}$ hair follicles consisted of groups of more than 2 hair shafts (Fig 2K and L). Together, these data suggest that genetic ablation of *Slc1a3* leads to reduced hair follicle stem cell activation and proliferation, consequently resulting in disturbed anagen initiation, impaired hair follicle cycling and, over time, reduced fur density."

Collectively, we suggest interpreting these results as the reviewer indicate. Young *Slc1a3*$^{-/-}$ mice are late to initiate anagen (as demonstrated both at p28 and at 3 months of age), and over time, aged mice will have undergone less hair cycles than *Slc1a3*$^{+/+}$ mice, resulting in a reduced number of club hair in each hair follicle.

4. When comparing the anagen and telogen regions within each aged animal, the authors write "we found increased proliferation of SG and IFE basal cells in Slc1a3+/+ mice during anagen, in line

with our data from young mice." (lines 70-71). However, the p-value for these data are 0.09 (Fig. 1r). Or did I misunderstand these data?

We thank the reviewer for pointing out this mistake. We have changed the sentence accordingly to: "Comparing anagen and telogen regions within each aged animal, we found increased proliferation of SG and IFE basal cells in *Slc1a3*$^{+/+}$ mice during anagen."

5. Please explain in more detail what exactly is shown in Figure 3a - this is challenging to follow, but would be important as it forms the basis for following experiments.

We thank the reviewer for pointing this out. Old Figure 3A is Figure 4A in the revised manuscript. We have now extended the description of the data in the text as following:

"To determine whether Slc1a3 expression is temporally regulated or continuously expressed in individual stem cells, we turned to the ORS, in which most cycling hair follicle stem cells are located, and mapped CreER^{T2} expression to cell position along the ORS (as quantified from the bulge to the hair follicle base). Comparing anagen stages IIIb, IIIc, IV and V, we found that the number of CreER^{T2} expressing cells increased together with the continuous growth of the follicle. While CreER^{T2+} cells could be localized starting from the first position below the bulge (+1) the density was highest in the middle of the ORS at all four stages (Fig 4A). This dynamic distribution of Slc1a3^{+} cells along the ORS offered a temporal resolution that we exploited."

We also modified the figure by adding horizontal lines to indicate the base of the HF for each anagen stage.

2nd Editorial Decision 19th Febuary 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal, and your patience with our response. Your revised manuscript has now been seen by the three original referees, whose comments are enclosed below. As you will see, all referees find that their concerns have been sufficiently addressed and are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues on data quantification (as to referee #3) as well as material & methods and formal formatting as outlined below, which need to be adjusted at re-submission.

REFEREE REPORTS

Referee #1:

The authors have successfully addressed all my concerns and the revisions have substantially improved the manuscript

Referee #2:

In this revised version authors have provided additional evidence and clarity around the key questions that I had raised.
The clarification and additional experiments around the timing of induction of lineage tracing and timing of collection, quantity of tamoxifen clearly supports the transient expression of slc1a3. This gives credit to the rasless experiment.
Moreover the use of inhibitors in slc1a3-/- mice is a great addition to prove the importance of the GluR functionally with slc1a3.
The only missing point of lesser importance would be a direct comparison to results published by Sada et al JID 2017 on the localisation of slc1a3 expression in the hair follicle to resolve some of the discord.

Referee #3:

My concerns on the manuscript have been addressed satisfactorily.

The result on reintroducing the Slc1a3 gene into cultured keratinocytes was quite surprising. It would be nice to report the extent of overexpression in this set-up. Perhaps in the future tuning the expression level and comparing the outcome of low vs. medium vs. high expression levels might be informative.

2nd Revision - authors' response 28th Febuary 2018

Referee #1:

The authors have successfully addressed all my concerns and the revisions have substantially improved the manuscript

We were happy to read that the reviewer found all concerns successfully addressed.

Referee #2:

In this revised version authors have provided additional evidence and clarity around the key questions that I had raised.
The clarification and additional experiments around the timing of induction of lineage tracing and timing of collection, quantity of tamoxifen clearly supports the transient expression of slc1a3. This gives credit to the rasless experiment.
Moreover the use of inhibitors in slc1a3-/- mice is a great addition to prove the importance of the GluR functionally with slc1a3.

We are pleased to have been able to address the reviewer's key questions to satisfaction.

The only missing point of lesser importance would be a direct comparison to results published by Sada et al JID 2017 on the localisation of slc1a3 expression in the hair follicle to resolve some of the discord.

We thank the reviewer for this valuable remark. We have now referenced the publication by Sada et al JID 2017 and commented on the differences of the two Slc1a3-CreER^{T2} mouse lines in the discussion as following:

"Using a different Slc1a3-CreER^{T2} mouse line, a recent study reported selective targeting of the inner root sheath in the anagen follicle (Sada, Jain et al., 2017). We could neither detect Slc1a3 nor CreER^{T2} expression in the inner root sheath but only in the outer root sheath, indicating that the line used by Sada et al., does not recapitulate the endogenous expression faithfully."

Referee #3:

My concerns on the manuscript have been addressed satisfactorily.

We were delighted to read that the reviewer's concerns have been resolved.

The result on reintroducing the Slc1a3 gene into cultured keratinocytes was quite surprising. It would be nice to report the extent of overexpression in this set-up.

The extent of Slc1a3 overexpression was determined by RT-qPCR and, in Figure 5R, presented as fold change over expression in uninduced (-Dox) keratinocytes.

Perhaps in the future tuning the expression level and comparing the outcome of low vs. medium vs. high expression levels might be informative.

We are thankful for the reviewer's valuable advice for future studies.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Christian Göritz
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-98280

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< 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 χ2 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?	No statistical methods have been used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See Methods, "Quantifications and Statistical Analysis".
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the analysis.
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.	
For animal studies, include a statement about randomization even if no randomization was used.	For pharmacological treatments Slc1a3-CreER x Rosa26-YFP animals were randomly assigned to either the experimental or control group.
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.	
4.b. For animal studies, include a statement about blinding even if no blinding was done	Quantitative analysis was not conducted in a blinded fashion.
5. For every figure, are statistical tests justified as appropriate?	Yes. Parametric statistical tests were use for normally distributed data and alternative nonparametric tests were used in case of non normally distributed data.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Sample size was too small to conduct statistical tests to assess normal distribution. Data was visually inspected.
Is there an estimate of variation within each group of data?	Data points in each experimental group are plotted. Data is shown as mean +/- SEM or + SEM. In data source files standard deviation is also reported.
Is the variance similar between the groups that are being statistically compared?	Data variance was compared with F-test in GraphPad Prism. In a few groups statistically compared the spread of data varied. In this case, t-tests were performed using welch correction in GraphPad prism.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See methods "Histology and Immunohistochemistry"

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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.	Reported in Materials and Methods and Excel files with source data.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures were carried out in accordance to the Swedish and European Union guidelines and approved by the institutional ethical committee (Stockholm Norra Djurförsöksetiska Nämnd).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	This study complies to the ARRIVE guidelines for animal studies.

E- Human Subjects

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

F- Data Accessibility

18: Provide 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'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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