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Polycomb subunits Ezh1 and Ezh2 regulate the Merkel cell differentiation program in skin stem cells

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

Editor: Anke Sparmann

1st Editorial Decision 11 January 2013

Thank you for submitting your research manuscript (EMBOJ-2012-84094) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate your study and are supportive of publication in The EMBO Journal. Nevertheless, referee #1 and #2 do raise a number of concerns that should be taken into consideration. Although this will entail some additional experimentation, many issues can be addressed by textual changes and clarifications of experimental procedures.

Given the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that attends to the raised concerns in full. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage. Please do not hesitate to contact me should any particular argument require further clarification.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

REFEREE COMMENTS

Referee #1

This manuscript addresses the molecular control of merkel cell development. Investigating mice lacking Ezh1/2 in skin keratinocytes, the authors find that merkel cell numbers are increased. They show that Sox2 is increased in the epidermis of Ezh1/2 embryos and that deletion of Sox2 in the skin results in a reduction/absence of merkel cells. Finally, deletion of Sox2 in Ezh1/2 conditional null mice results in loss of merkel cells. These data identify a molecular pathway involving Ezh1/2 and Sox2 that controls Merkel cell development.

This is a beautiful study identifying novel mechanisms that regulate merkel cell development. The use of several mouse models used to test the authors' hypotheses is impressive and the data have far-reaching consequences for our understanding of epidermal lineage commitment and cancer. The manuscript is generally clearly written and nicely reflects the literature in the field. Below, I list suggestions for further improvements and after addressing these issues, this work will be appropriate for publication in the EMBO J.

Major issues
1. Throughout the manuscript, the authors use the term epidermal stem cells to describe epidermal keratinocytes. This term seems to be used inaccurately to describe the cells that make up the epidermal basal layer. Similarly, the keratin 5 is not an epidermal stem cell marker as described on page 6 but rather a proliferative keratinocyte marker.

2. In Figure 2B, there appears to be a K18+ nucleus that is not GFP+. Does this indicate that all merkel cells are not derived from K14+ cells? Related to this point, the authors should reference Woo et al. 2010, Morrison et al 2009 and Van Keymeulen et al 2009 when describing the epithelial origin of Merkel cells on page 7.

3. The conclusion that Ezh1/2 regulates the differentiation of merkel cells is not fully supported by the data, rather the data show that Ezh1/2 and Sox2 control merkel cell development. The exact mechanism (progenitor proliferation/differentiation/apoptosis) as to how Ezh1/2 or Sox2 regulates merkel cell number is still unclear.

The authors conclude that loss of Ezh1/2 does not alter the proliferative status of Merkel cells, however they do not analyze stages of skin development when Merkel cells (or their progenitors) are proliferative. Perhaps Ezh1/2 regulates the proliferation of merkel cell progenitor cells prior to P0 or without altering Ki67 expression.

The same concern exists for the analysis of apoptosis in the Sox2cKO mice. The authors analyze apoptosis in the skin without any indication that merkel cells exist at the timepoint analyzed. Do the Sox2cKO mice display enhanced apoptosis in the skin at E14 or E15 prior to specification of merkel cells?

Also, the analysis of merkel cell differentiation is not clear. The authors state that merkel cells appear "only by E17" in WT mice but show images of merkel cells at E16 in WT mice in Figure 2E. Does merkel cell development display regional differences in the embryo? If so, the authors should be sure to analyze the same regions of the embryo to define the timing of merkel cell development.

A direct role for Sox2 in directing merkel cell differentiation is suggested by the experiments overexpressing Sox2 in keratinocytes. Are other merkel cell genes induced or only Atoh1?

The use of Sox2 to identify Merkel cells is suggested by the data. Can the authors analyze the timing of Sox2 expression and Atoh1 or Isl1 to determine the timing of its expression compared to these Merkel cell genes?

Given how little is known about how the merkel cell lineage forms, the authors may be able to alter their conclusions of the presented data and address these other possibilities in the discussion.

4. The comparison of gene expression in Ezh1/2 null epidermal cells and Merkel cells is not clear, which may result from an incomplete description of the analysis in the results section. What cell populations were used for each gene expression profiling experiment? If Merkel cell numbers are increased in the Ezh1/2 null mice, why is K20 not upregulated? The fact that other classic Merkel cell genes are upregulated is not clear from the results but is in the Supplemental Table.

5. The analysis of Atoh1GFP mice is confusing when compared to the staining for other Merkel cell markers since there are Atoh1GFP+ cells in the Sox2cKO but histological analysis of Merkel cell markers shows a complete absence of merkel cells. Can the authors show histology of the Atoh1GFP Sox2cKO mice? Is this discrepancy because Atoh1 is expressed prior to Rab3c, K18 or K20?

Related to this point, the authors state that Atoh1GFP signal intensity is decreased in the Sox2cKO. However, the data presented suggests that the number of cells is reduced, not the intensity of GFP expression. Likewise, the fluorescent intensity for the Isl1 expression (SFig4) is not as informative as a quantification of the number of cells. These data also suggest that Atoh1 and Isl1 may be expressed prior to Sox2 during Merkel cell development (see point 3).

6. The direct regulation of polycomb action on Sox2 expression is also not definitive from the data presented since the authors do not show that Ezh1/2 directly binds to the Sox2 promoter and loss of Ezh1/2 alters the H3K27me3 of the Sox2 promoter. Without these data, it is possible that Ezh1/2 indirectly regulates genes that controls Sox2 expression.

Minor comments:
The description of merkel cell development in the introduction does not flow well.

The color labels of SFig4fA seem to be switched-is beta4 integrin in red?

Page 11 in the sentence: "The collected evidence in this work suggests a mechanistic model for Polycomb repression of the Merkel cell lineage in epidermal stem cells": collected should be collective.

In the methods, transmission EM section: insure should be ensure

In Fig.1, 2, 4C, 4D and 5, what is the n (number of cells) quantified in each quantification?

Referee #2

The authors found that the number of merkel cells (MC) is increased in the IFE of the whisker as well as in dorsal and ventral skin of Ezh1/2 DKO mice. MC from Ezh1/2 DKO seem identical to WT MC: they are innervated by the same sensory fibers (NF200+); they express the same presynaptic machinery (Rab3C+), they express the same type of keratins (K20+, K18+), and have the same cytoplasmic neuroendocrin granules (EM).
Then, the authors try to understand the mechanisms underlying the excess of MC in Ezh1/2 DKO mice. In the Ezh1/2 DKO mice, MC have lost H3K27me3 activity as the other epidermal cells and are traced by an epidermal lineage tracing (K14 H2BGFP), suggesting that they arise from an embryonic epidermal progenitor. They are negative for Ki67 staining and do not incorporate BrdU showing that those MC are postmitotic and do not divide to increase their number.

By analyzing microarray data from Ezh1/2 DKO, The authors showed that Ezh1/2 regulate Sox2 expression in MC. They confirmed using Chip experiment that Sox2 promoter region is trimethylated at K27 in normal epidermis. They confirmed that in Ezh1/2 DKO developing epidermis, Sox2 expression is upregulated. Immunofluorescence (IF) experiment reveals that early specified MC in WT epidermis express sox2 and that Sox2 expressing cells are expanded in Ezh1/2 DKO epidermis.

To assess the role of Sox2 in MC development, the authors performed Sox2 loss of function in Ezh1/2 DKO (Sox2/Ezh1/2 TKO) in the epidermis and show that Sox2 loss of function decreased

the excess of MC cells seen in Ezh1/2 DKO epidermis. This reinforces the hypothesis of Sox2 role in MC specification. In Sox2/Ezh1/2 TKO DKO epidermis, they showed that Math1 is decreased by 60%, suggesting that Sox2 may regulate Math1. Sox2/Ezh1/2 TKO DKO mice present a decrease in the number of Math1-GFP cells in the epidermis. Using cultured epidermal cell system they showed that Sox2 overexpression results in the increase of Math1 mRNA. Moreover, they demonstrate that Sox2 directly bind Math1 enhancer region of the promoter using Chip experiment, confirming the direct role of Sox2 in Math1 regulation (they did the same observation for another MC transcription factor Isl1). They proposed a model of MC specification in which Ezh1/2 regulate Sox2 activity, which in turn regulate Math1 expression that finally trigger MC specification.

General comments:
- This is a very interesting manuscript, the general message of the paper is clear and the data shown are convincing and support the message of the study. I believe this manuscript will be interesting for the reader of EMBO J.
- However some points need to be clarified and some new data will be required to better substantiate some of the claims of this paper.
- The choice of not studying MC of the paw epidermis and whisker follicles is difficult to understand since it is the body locations where they are by far the most abundant in mice epidermis. Do Ezh1/2 KO present excess of merkel cells in these body locations? What is the role of Sox2 in regulating MC specification at these sites.
- Some of the experimental procedures are not sufficiently detailed
(FACS gating, qPCR performed on total epidermis or sorted cells (e.g. fig4F)?).

Specific comments:
Figure 1:
- The authors need to provide more details about how quantifications were performed: what is the number of MC scored in WT and Ezh1/2 DKO? What is the total length of epidermis quantified in WT and Ezh1/2 DKO?
- The authors state that they assess the number of MC in P90 Ezh1/2 DKO epidermis. No IF of P90 mice are presented in main or supplemental figures, only the quantification. Furthermore, the P90 quantifications have been made on ventral skin, back skin and IFE cells of the whisker pad. The regions enriched for MC in adult mice are the paw epidermis and whisker follicles. The authors need to assess the number of MC in those regions.
- Idem for developing mice. From E15, whisker follicles start to develop. At that time developing MC are K8+K20- and become K8+K20+ at E17. In paw epidermis, K8+K20- developing MC are observed at E17, and K8+K20+ MC are found at P1.
- To prove that MC are functional, the authors should perform FMI43X dye uptake .
- The authors should perform statistical analysis.

Figure 2:
- Same comment than for Fig1: Present the number of MC scored.
- There is no clear protocol to understand how the fluorescent intensity in Fig2A and B (right panels) is quantified?
- The authors need to give more details about the BrdU pulse experiment: is it a short or a long pulse? What is the time between BrdU administration and analysis?
- MC appearance: The authors have to specify the epidermis area analyzed in the Fig2 E and F.
- Pictures illustrating supplemental Fig2 E and F are missing (touch dome quantification). The data for the whisker follicles need to be presented.
- The data proposed to show that the number of MC cluster is increased in Ezh1/2 DKO compared to WT is not relevant (supp Fig2F). How do the authors define MC cluster? If they are refereeing to touch dome (TD), they need to present a staining showing TD of the IFE (CD200, see Woo, 2010, Development) and show an increase in the number of TD stained and observed.

Figure3:
- What is the proportion of cells that are committed toward MC lineage in Ezh1/2 DKO?
- In Fig 3E-F and supp Fig3, the author suggest that the keratinocyte Sox2+ observed will become MC. This statement should be accompanied by a quantification of the number of Sox2+K20- at from E15 to P0 to show that this population decreases, comforting the idea that Sox2 is an early marker of MC.

- Is Sox2 expression localized sporadically in the IFE or is it located in MC cluster (TD)?

Figure 4:
- The authors do not specify the area of the skin where the MC quantification has been performed. The whisker follicles and paw epidermis should be quantified.
- In Sox2 loss of function, the number of MC scored and the length of epidermis quantified have to be specified.
- in Sox2 loss of function, there is a significant difference in the cells positive for K18 and K20. Can the author comment on that? .
- No quantification of the Rab3C + cells in Sox2 loss of function (Supp Fig4).
- Active caspase 3 immunostaining has been performed at P2. If the MC are not specified because of an increase of cell death, the authors should assess activated caspase3 much more earlier (around the moment of MC specification: E16, E17, E18). Moreover, the authors have to take into account the possibility that the MC progenitors could die. Besides, there is not positive control for caspase 3 staining.
- There is no quantification for the number of MC in Sox2 loss of function during adulthood (supp Fig4).
- Fig4F and supp Fig4L: the authors should specify if the qPCR data presented have been performed on total skin or sorted cells, the cell isolation protocol, and the eventual gating used to sort the cells.
- Fig4G: what combination of antibodies do the authors use to gate the basal epidermis?
- Math1-GFP is only partially decreased in Sox2cKO mice suggesting that other mechanisms beside Sox2 control Math1 expression and MC specification. This point should be clearly discussed.

Figure 5:
- Fig5A right panel: the quantification of the MC number in Sox2/Ezh1/2 TKO mice is missing.
- supp Fig5G: there is a discrepancy in the number of MC observed depending on the marker used to detect them (K18, K20, Rab3c). The authors need to present the number of MC quantified and the length of epidermis quantified for each time point. They should also demonstrate the statistical relevance of their data for each condition.
- Supp Fig5H and I: the whiskers IFE is analyzed but not the follicle. What about the number of MC in Sox2/Ezh1/2 TKO whisker follicles and paw epidermis?

Referee #3
This study investigates the important subject of the role of the key epigenetic regulator, the polycomb complex, in tissue stem cells, using the epidermis as a model. The introduction is clear and concise, but a summary of the EZH1/2 knockout epidermal stem cell and hair follicle phenotypes reported previously and a cartoon to show the how the Merkel cell (MC) lineage is related to other epidermal lineages might help a general reader.

Fig 1 presents clear evidence that EZH1/2 null epidermis has increased numbers of MC, positive for markers and by EM. Functional analysis has not been performed, but as the focus of the paper is on lineage determination this is perhaps not essential.

In Fig 2 the H3K27me3 mark, absent from mutant keratinocytes, is also found to be missing in MC, consistent with MC being keratinocyte lineage derived. A lineage tracing experiment with a histone-GFP transgene expressed from a Krt14 promoter confirms the common origin of MC and keratinocytes and cell cycle staining demonstrates that MC are post mitotic in wild type and null epidermis, so the increased number of MC is not due to a failure of cycle exit. Interestingly MC appear earlier in null than in wild type epidermis (E16 vs 17). The increase in the number of MC clusters in null skin supports the hypothesis that the mutation affects lineage determination.

Fig 3 examines differential gene expression comparing genes expressed in MC in wild type and null epidermis. Sox2 emerges as a highly differentially expressed transcript. That Sox2 is directly regulated by PRC is confirmed by the presence of H3K27me3 marks at the Sox2 gene. Sox2 is expressed in MC and appears in embryonic skin just prior to the first MC.

Fig 4 demonstrates that MC are depleted in Sox2 null epidermis. This phenotype is associated with a decrease in expression of Atoh1, an essential determinant of MC differentiation. Sox2 is shown to directly regulate the Atoh1 gene.

In Fig5 An epistasis experiment in EZH1/2 Sox2 triple null mice is performed. These animals are depleted of MC consistent with the author's hypothesis that loss of PRC repression leads to activation of Sox2, which in turn promotes transcription of Atoh1, leading to Merkel cell differentiation

This is a well written and clearly presented paper, where a series of high quality experiments are used to provide robust support for a novel pathway directing MC differentiation. The work will be of interest to stem cell biologists as well as MC and epidermal specialists and is appropriate for publication in the EMBO Journal.

1st Revision - authors' response 02 April 2013

Referee #1

This manuscript addresses the molecular control of merkel cell development. Investigating mice lacking Ezh1/2 in skin keratinocytes, the authors find that merkel cell numbers are increased. They show that Sox2 is increased in the epidermis of Ezh1/2 embryos and that deletion of Sox2 in the skin results in a reduction/absence of merkel cells. Finally, deletion of Sox2 in Ezh1/2 conditional null mice results in loss of merkel cells. These data identify a molecular pathway involving Ezh1/2 and Sox2 that controls Merkel cell development.

This is a beautiful study identifying novel mechanisms that regulate merkel cell development. The use of several mouse models used to test the authors' hypotheses is impressive and the data have far-reaching consequences for our understanding of epidermal lineage commitment and cancer. The manuscript is generally clearly written and nicely reflects the literature in the field. Below, I list suggestions for further improvements and after addressing these issues, this work will be appropriate for publication in the EMBO J.

We are grateful to the reviewer for the insightful comments and suggestions and excited to see that he/she finds our work to be appropriate for the EMBO J. We have addressed the reviewer's comments and posted our responses below.

Major issues
1. Throughout the manuscript, the authors use the term epidermal stem cells to describe epidermal keratinocytes. This term seems to be used inaccurately to describe the cells that make up the epidermal basal layer. Similarly, the keratin 5 is not an epidermal stem cell marker as described on page 6 but rather a proliferative keratinocyte marker.

A recent paper from Dr. Cedric Blanpain's laboratory (Mascré et al 2012, Nature) describes the presence of stem and progenitor cells in the basal layer of the epidermis. Since no markers to discriminate stem vs progenitor cells are currently available we have no tools to specifically address this point. We thus have adapted the text accordingly [For example, on Page 6: As expected, at P0 all Merkel cells co-express K18 and K20 (Figure 1C) and in accordance with previous reports (Maricich et al, 2009; Morrison et al, 2009; Van Keymeulen et al, 2009; Woo et al, 2010), they lose expression of epidermal **progenitor** marker K5 (Figure 1D)].

2. In Figure 2B, there appears to be a K18+ nucleus that is not GFP+. Does this indicate that all merkel cells are not derived from K14+ cells? Related to this point, the authors should reference Woo et al. 2010, Morrison et al 2009 and Van Keymeulen et al 2009 when describing the epithelial origin of Merkel cells on page 7.

Indeed, as the reviewer pointed out, the some of the K14-cre derived GFP+ cells appear to be weaker. We believe that this is due to dilution of the GFP+ protein that is expressed in the K14 +

basal cells but not in the progeny of these cells, that do not express K14. We performed careful analysis of the K14-GFP signal in K18+ cells of Ezh1/2 2KO and WT Merkel cells. We found that all of the K18+ cells are indeed GFP+, including those mentioned by the referee, whereas dermal and melanocyte cells are GFP negative. Quantifications are provided in Figure 2B right. The GFP level in WT and Ezh1/2 2KO K18(+) cells was significantly lower than in the K14-expressing basal epidermal cells, but comparable to the level seen in K14-derived suprabasal cells (Figure 2B, right) indicating that Ezh1/2 2KO Merkel cells are K14-derived and therefore epithelial in origin. To further illustrate our point, we have included the GFP single channel images that correspond to Figure 2B in SFigure2C, which should clearly show that K18(+) cells are GFP+ in WT and Ezh1/2 2KO skin.
We added mentioned references to the paper text [Page 7: Together these data indicate that Ezh1/2 2KO Merkel cells are indeed K14-derived and therefore epithelial in origin, consistent with previous reports (Morrison et al, 2009; Van Keymeulen et al, 2009; Woo et al, 2010)].

3. The conclusion that Ezh1/2 regulates the differentiation of merkel cells is not fully supported by the data, rather the data show that Ezh1/2 and Sox2 control merkel cell development. The exact mechanism (progenitor proliferation/differentiation/apoptosis) as to how Ezh1/2 or Sox2 regulates merkel cell number is still unclear.

We have now included a more detailed analysis of how Ezh1/2 and Sox2 regulate proliferation/differentiation/apoptosis of Merkel cells. These points are addressed below.

The authors conclude that loss of Ezh1/2 does not alter the proliferative status of Merkel cells, however they do not analyze stages of skin development when Merkel cells (or their progenitors) are proliferative. Perhaps Ezh1/2 regulates the proliferation of merkel cell progenitor cells prior to P0 or without altering Ki67 expression.

The vast majority of literature has shown that Merkel cells are postmitotic (REF). However, we analyzed Merkel cell proliferation in WT and 2KO skins by Ki67 expression and BrdU incorporation. We analyzed back skin at E16 (first appearance of fully differentiated Merkel cells in 2KO skin) (Figure 2C-D, SFigure 2D), P0 (SFigure 2E) and P14 (SFigure 2F), and whisker and paw regions at P0 (SFigure 2G and H), where we clearly show that K18+ and K20+ Ezh1/2 2KO Merkel cells are Ki67- and BrdU-negative. Our data indicate that these cells are non-proliferative throughout stages of Merkel cell lineage specification and in accordance with the published works.
To date there is a single paper (Woo et al 2010 Development) that analyzed the presence of Merkel cell progenitor cells in mouse skin. Woo et al showed that in adult mice CD200+ cells are located in direct contact with Merkel cells and transplantation experiments showed that CD200+ cells were required to regenerate both epidermis and Merkel cells.
We contacted Dr. Owens, the senior author on the paper, and obtained the CD200 IF protocol and antibody information for the data shown in Woo et al 2010. Unfortunately, we were unable to reproduce this staining in our P0 samples, or in WT adult mice as shown in the Woo et al paper. We also attempted this using a second CD200 antibody with both the protocol provided by Dr. Owens and the protocol routinely used in our lab but once again we were unable to obtain a conclusive staining. We have included the obtained images at the end of this letter (A and B). Thus without the CD200 staining we are unable to analyze proliferation of Merkel progenitor cells in the current study.

The same concern exists for the analysis of apoptosis in the Sox2cKO mice. The authors analyze apoptosis in the skin without any indication that merkel cells exist at the timepoint analyzed. Do the Sox2cKO mice display enhanced apoptosis in the skin at E14 or E15 prior to specification of merkel cells?

To fully address this point we first analyzed when fully differentiated Merkel cells appear in WT skin and found that the first K20+ cells appear at E17 (Figure 2F and 3E). We next analyzed WT/Sox2cKO skin at E15 and E16, ages that cover the point prior to specification of Merkel cells, and did not detect an increase in apoptosis in the skin of Sox2cKO mice (SFigure 4K-L). We performed similar analysis of whisker and paw regions and also did not observe an increase of cell death in Sox2-null mice (SFigure 4N-S). The appearance of rare apoptotic cell in WT skin was observed and served as a positive control for activated-caspase 3 IF staining (SFigure 4L).

Also, the analysis of merkel cell differentiation is not clear. The authors state that merkel cells appear "only by E17" in WT mice but show images of merkel cells at E16 in WT mice in Figure 2E. Does merkel cell development display regional differences in the embryo? If so, the authors should be sure to analyze the same regions of the embryo to define the timing of merkel cell development.

To address the question of seemingly conflicted data on time of Merkel cell appearance, we carried out a thorough temporal analysis and spatial analysis of the different Merkel cells markers during development. We did not notice any regional differences in the specification of Merkel cells. However, we did observe temporal differences of expression of Merkel cell differentiation genes. We found that Sox2+ and Atoh1-GFP+ first appear at E15-E16, followed by K18 and K20 at E16.5 and E17.5, respectively (Figure 5E). Thus while some makers can be detected as early as E15, fully differentiated Merkel cells appear only at E17 in WT skin.
In E16 WT skin we observed very few K18+ cells and no K20+ cells (Figure 2E,F). However, in Ezh1/2 2KO at E16 we detected the presence of both K18+ and K20+ cells (Figure 2E,F). These data thus indicate that there is acceleration in Merkel cell development in 2KO skin compared to WT and is discussed in the new version of the manuscript on page 7-8.

A direct role for Sox2 in directing merkel cell differentiation is suggested by the experiments overexpressing Sox2 in keratinocytes. Are other merkel cell genes induced or only Atoh1?

We have now provided expression analysis for several Merkel cell genes. RT-QPCR analysis showed a small upregulation of K20 in WT cells overexpressing Sox2, but expression of other Merkel cell markers remained unchanged. This new data is now presented in Figure 5C.

The use of Sox2 to identify Merkel cells is suggested by the data. Can the authors analyze the timing of Sox2 expression and Atoh1 or Isl1 to determine the timing of its expression compared to these Merkel cell genes?

We analyzed the onset of Sox2 and Atoh1 expression by performing IF studies on Atoh1-GFP skins collected from E13 to E17. We found the first appearance of a few Sox2+ and Atoh1-GFP+ cells in WT skin at E15.5 (Figure 5E), well before the expression of K20 at E17 (Figure 5F). We were unable to find embryos that showed Merkel cells expressing only Sox2 or Atoh1-GFP in epithelium. It is likely that the onset of these genes occurs in a small window that cannot be resolved through this type of analysis due to the limitations of mouse mating.

4. The comparison of gene expression in Ezh1/2 null epidermal cells and Merkel cells is not clear, which may result from an incomplete description of the analysis in the results section. What cell populations were used for each gene expression profiling experiment? If Merkel cell numbers are increased in the Ezh1/2 null mice, why is K20 not upregulated? The fact that other classic Merkel cell genes are upregulated is not clear from the results but is in the Supplemental Table.

The detailed purification method, FACS protocol, microarray hybridization and analysis of WT and Ezh1/2 2KO basal cells was published in Ezhkova et all Genes & Development 2011 paper and here we analyze published microarray results. FACS gating was done on live $CD140a^{-}CD207^{-}CD117^{-}CD31^{-}Sca1^{+}\alpha6\text{-integrin}^{high}$ cells to exclude many non-epithelial cell types. This information is now included in the Methodology section of the manuscript. We also included a table showing raw numbers of hybridization signals of total cDNAs isolated from WT and Ezh1/2 2KO basal cells for top three genes expressed in Merkel cells (SFigure3A). Please note that none of the genes are upregulated in 2KO epidermis suggesting the purity of FACS-isolated populations and confirming the selective activation of a few Merkel genes in 2KO basal cells.
Our analysis of gene expression data revealed that only 10% of Merkel signature genes are unregulated in Ezh1/2 2KO basal cells, indicating that there is a selective activation of a subset of Merkel cell genes in 2KO basal cells.

5. The analysis of Atoh1GFP mice is confusing when compared to the staining for other Merkel cell markers since there are Atoh1GFP+ cells in the Sox2cKO but histological analysis of Merkel cell markers shows a complete absence of merkel cells. Can the authors show histology of the Atoh1GFP Sox2cKO mice? Is this discrepancy because Atoh1 is expressed prior to Rab3c, K18 or K20?

We agree with the reviewers that this is an important point to clarify, which we have tried to do to maximum extent, given some technical limitations.

First, we analyzed the temporal expression of Atoh1 relative to other Merkel cell markers. We initially attempted to use commercial antibodies to Atoh1, but were ultimately unsuccessful. Without good antibodies available, we analyzed expression of Atoh1 using Atoh1-GFP mice (obtained from Jax lab), a knock-in mouse strain where Atoh1 is tagged at 3'end with GFP. This analysis revealed that Atoh1-GFP+ cells first appear at E15.5, when the first Sox2+ cells are observed in epithelium (Figure 5E), but prior to the expression of K20 that is observed at 17 (Figure 5F). Thus indeed Atoh1-GFP is expressed prior to markers of fully differentiated Merkel cells.

Second, we investigated if K14-Cre-mediated excision occurs prior to initial Sox2 expression as it would explain the appearance of Atoh1+ cells in Sox2cKO. To do that we crossed K14-Cre mice with Rosa-ActB-tdTomato,-eGFP mice (Jax stock no 007576), in which cells turn from red to green upon Cre activity. The analysis revealed that at E14 cells of back skin, whiskers and paws are GFP+, consistent with strain data provided by Jax (data are included at the end of the rebuttal letter; C). We also did not observe mosaicism in GFP expression. Thus by E15 (when first Sox2+ and Atoh1-GFP+ cells appear), K14-mediated Cre excision of Sox2 has occurred. We confirmed these data by performing IF studies of Sox2 expression at E16 in WT/Sox2cKO mice. In Sox2cKO mice, Sox2+ cells were only observed in the dermal papilla (DP), mesenchymal cells that are not targeted by K14-Cre strategy, whereas in WT we observed Sox2+ cells in both DP and basal layer.

Third, we performed histological analysis of Atoh1-GFP Sox2cKO mice (SFigure5A) and observed a drastic reduction in the number of K20+ cells indicating that Sox2cKOAtoh1-GFP mice have loss of the fully differentiated Merkel cells as observed in Sox2cKO mice.

Finally, we analyzed the presence of Atoh1-GFP+ cells in adult Sox2cKO mice. At P35 there was a complete absence of Atoh1-GFP+ cells, whereas they were still present in WT (Figure 5G).

Combining these data together, we speculate that during Merkel cell specification the loss of Polycomb repression leads to an initial low level of Atoh1 expression, consistent with previously published data showing that *Atoh1* is a Polycomb target gene (Lien et al Cell Stem Cell 2011 and Ezhkova Genes and Development 2011). However, this initial Atoh1 expression cannot be sustained or promoted without the function of the Sox2 transcription factor. Additionally, the Atoh1-GFP fusion protein may have a lower turnover rate than endogenous Atoh1, resulting in the presence of a few Atoh1-GFP+ cells in Sox2cKO skin at P0. We addressed this point in the discussion section of the main text.

Related to this point, the authors state that Atoh1GFP signal intensity is decreased in the Sox2cKO. However, the data presented suggests that the number of cells is reduced, not the intensity of GFP expression. Likewise, the fluorescent intensity for the Isl1 expression (SFig4) is not as informative as a quantification of the number of cells.

We included data showing a decrease in the number of Isl1+ cells in Sox2cKO skin and these data are presented in SFigure5E. Thus there is both a decrease in the number of Isl1+ and Atoh1-GFP+ cells as well as a decrease in fluorescent intensity of Atoh1 and Isl1 in remaining Atoh1-GFP+ and Isl1+ cells.

These data also suggest that Atoh1 and Isl1 may be expressed prior to Sox2 during Merkel cell development (see point 3).

As discussed in the point 3, we speculate that loss of Polycomb repression induces initial expression of Atoh1 however Sox2 is required to promote Atoh1 expression. This hypothesis is consistent with 1) the Polycomb targeting of the Atoh1 gene (as previously published in Ezhkova et al Gene and Development 2011 and Lien et al Cell Stem Cell 2011 and also shown in SFigure5C), and 2) complete loss of Atoh1-GFP+ cells in Sox2cKO mice at P35 (Figure 5G).

6. The direct regulation of polycomb action on Sox2 expression is also not definitive from the data presented since the authors do not show that Ezh1/2 directly binds to the Sox2 promoter and loss of Ezh1/2 alters the H3K27me3 of the Sox2 promoter. Without these data, it is possible that Ezh1/2

indirectly regulates genes that controls Sox2 expression.

We performed ChIP analysis and included H3K27me3 data in Figure 3D and Ezh1 and Ezh2 data in SFigure 3B. These data show the presence of Ezh1 and Ezh2 at the Sox2 gene and loss of H3K27me3 signal at Sox2 in Ezh1/2 2KO vs WT. This data strongly suggests a direct regulation of *Sox2* by the activity of Ezh1/2 proteins.

Minor comments:
The description of merkel cell development in the introduction does not flow well.

We have modified the Introduction.

The color labels of SFig4fA seem to be switched-is beta4 integrin in red?

We thank the reviewer for pointing this out. This figure has been restructured and labels have been corrected as necessary.

Page 11 in the sentence: "The collected evidence in this work suggests a mechanistic model for Polycomb repression of the Merkel cell lineage in epidermal stem cells": collected should be collective.

We believe the text to be correct as is and will defer to the editorial judgment of the EMBO Journal staff.

In the methods, transmission EM section: insure should be ensure

We corrected this.

In Fig.1, 2, 4C, 4D and 5, what is the n (number of cells) quantified in each quantification?

We included the total number of analyzed cells (n) and total length of analyzed skin region into figure legends.

Referee #2

The authors found that the number of merkel cells (MC) is increased in the IFE of the whisker as well as in dorsal and ventral skin of Ezh1/2 DKO mice. MC from Ezh1/2 DKO seem identical to WT MC: they are innervated by the same sensory fibers (NF200+); they express the same presynaptic machinery (Rab3C+), they express the same type of keratins (K20+, K18+), and have the same cytoplasmic neuroendocrin granules (EM).
Then, the authors try to understand the mechanisms underlying the excess of MC in Ezh1/2 DKO mice. In the Ezh1/2 DKO mice, MC have lost H3K27me3 activity as the other epidermal cells and are traced by an epidermal lineage tracing (K14 H2BGFP), suggesting that they arise from an embryonic epidermal progenitor. They are negative for Ki67 staining and do not incorporate BrdU showing that those MC are postmitotic and do not divide to increase their number.

By analyzing microarray data from Ezh1/2 DKO, The authors showed that Ezh1/2 regulate Sox2 expression in MC. They confirmed using Chip experiment that Sox2 promoter region is trimethylated at K27 in normal epidermis. They confirmed that in Ezh1/2 DKO developing epidermis, Sox2 expression is upregulated. Immunofluorescence (IF) experiment reveals that early specified MC in WT epidermis express sox2 and that Sox2 expressing cells are expanded in Ezh1/2 DKO epidermis.

To assess the role of Sox2 in MC development, the authors performed Sox2 loss of function in Ezh1/2 DKO (Sox2/Ezh1/2 TKO) in the epidermis and show that Sox2 loss of function decreased the excess of MC cells seen in Ezh1/2 DKO epidermis. This reinforces the hypothesis of Sox2 role in MC specification. In Sox2/Ezh1/2 TKO DKO epidermis, they showed that Math1 is decreased by 60%, suggesting that Sox2 may regulate Math1. Sox2/Ezh1/2 TKO DKO mice present a decrease in

the number of Math1-GFP cells in the epidermis. Using cultured epidermal cell system they showed that Sox2 overexpression results in the increase of Math1 mRNA. Moreover, they demonstrate that Sox2 directly bind Math1 enhancer region of the promoter using Chip experiment, confirming the direct role of Sox2 in Math1 regulation (they did the same observation for another MC transcription factor Isl1). They proposed a model of MC specification in which Ezh1/2 regulate Sox2 activity, which in turn regulate Math1 expression that finally trigger MC specification.

General comments:
- This is a very interesting manuscript, the general message of the paper is clear and the data shown are convincing and support the message of the study. I believe this manuscript will be interesting for the reader of EMBO J.
- However some points need to be clarified and some new data will be required to better substantiate some of the claims of this paper.
- The choice of not studying MC of the paw epidermis and whisker follicles is difficult to understand since it is the body locations where they are by far the most abundant in mice epidermis. Do Ezh1/2 KO present excess of merkel cells in these body locations? What is the role of Sox2 in regulating MC specification at these sites.
- Some of the experimental procedures are not sufficiently detailed
(FACS gating, qPCR performed on total epidermis or sorted cells (e.g. fig4F)?).

We are grateful to the reviewer for finding our paper to be interesting for the readers of the EMBO J. We are thankful to the reviewer for pointing out to some clarifications of data and missing information on the experimental procedures. We have now included this information in text. We also analyzed the presence of Merkel cells in paws and whiskers follicles and included these data. Below we have addressed the reviewer's comments point by point.

Specific comments:

Figure 1:
- The authors need to provide more details about how quantifications were performed: what is the number of MC scored in WT and Ezh1/2 DKO? What is the total length of epidermis quantified in WT and Ezh1/2 DKO?

We now included this information in the figure legends, Material and Methods, and Supplementary Table 2 for each quantification provided. Due to the highly variable number of Merkel cells between genotypes, we have included the total length of skin quantified in to the figure legends. We also provide complete information on total number of Merkel cell counted for each staining in the Supplementary Table 2.

- The authors state that they assess the number of MC in P90 Ezh1/2 DKO epidermis. No IF of P90 mice are presented in main or supplemental figures, only the quantification. Furthermore, the P90 quantifications have been made on ventral skin, back skin and IFE cells of the whisker pad. The regions enriched for MC in adult mice are the paw epidermis and whisker follicles. The authors need to assess the number of MC in those regions.

The Ezh1/2 2KO mice die at P0, as was described previously in Ezhkova 2011 Genes and Development. Consequently, all the quantifications for the number of Merkel cells at P90 were performed on back skins grafted onto nude mice. Thus, we cannot provide images and quantifications for P90 whisker and paws region as it is technically very challenging to graft these regions. We included P90 images of back skin grafts for WT/2KO to support quantifications (now presented in Supplemental Figure 1A).
Additionally, we have now performed the analysis of Merkel cells in paw and whisker regions of P0 WT and Ezh1/2 2KO mice and included these data in SFigure 1C-E. Similarly to the back skin data, we observed an increase in the number of Merkel cells in whisker IFE and paws in 2KO mice compared to WT (SFigure 1F). Interestingly, we have not observed an increase in Merkel cell number in 2KO whisker follicles. Quantifications showed that the number of Merkel cells in the whisker follicles is >8 times higher than in whiskers IFE, paws or back skin. We speculate that the whisker follicles are saturated with Merkel cells in WT mice, and thus the number cannot be increased in that region.

- Idem for developing mice. From E15, whisker follicles start to develop. At that time developing MC are K8+K20- and become K8+K20+ at E17. In paw epidermis, K8+K20- developing MC are observed at E17, and K8+K20+ MC are found at P1.

We thank the reviewer for providing the description of Merkel cell development at different body regions. We attempted to address these questions with the materials that we could obtain. As discussed above due to lethality of Ezh1/2 2KO pups, we were unable to obtain paws from P1 2KO animals. We analyzed P0 WT/2KO back skin, whiskers and paws for all genotypes and included data in SFigure1 A-F (2KO), SFigure4J-N (Sox2cKO), and SFigure6K-O (3KO). All the results are consistent with the conclusion drawn from the analysis of the back skin region in the previous version of the manuscript.

- To prove that MC are functional, the authors should perform FMI43X dye uptake.

We attempted to perform FMI43x dye uptake experiments as described in Van Keymeulen et al 2009. However, due to the neonatal death of 2KO/3KO pups we tried to adapt the described protocol to our model: First, the dye could not be incubated for 24h as described and had to be reduced to 4-6 hours; Second, the FMI43X dye injection was performed intradermal instead of intraperitoneal, which is not possible on P0 pups due to their size. We first aimed to validate this adapted FMI43x protocol in P0 Atoh1-GFP mice, but unfortunately the dye does not seem to accumulate specifically in Merkel cells, as we observed some staining around Atoh1-GFP negative areas. Representative images are shown at the end of this letter (D). It is likely that short dye incubation and dermal injection impede proper dye uptake, and thus prevent us from performing this analysis. We however believe that the provided electron microscopy data address the issue of functionality, as the structures used to identify Merkel cells by EM (neuroendocrine granules) are an indicator of the ability to execute neuroendocrine functions.

- The authors should perform statistical analysis.

We expanded our original statistical analysis on all quantifications and indicated the corresponding p values and skin length in figure legends.

Figure 2:
- Same comment than for Fig1: Present the number of MC scored.

We have now included the total length of skin quantified into the figure legends. We also provide complete information on total number of Merkel cell counted for each staining in the Supplementary Table 2.

- There is no clear protocol to understand how the fluorescent intensity in Fig2A and B (right panels) is quantified?

The protocol is now included in the Materials and Methods.

- The authors need to give more details about the BrdU pulse experiment: is it a short or a long pulse? What is the time between BrdU administration and analysis?

A standard 4h BrdU pulse was performed throughout the paper. The methods section has been expanded to reflect this more clearly.

- MC appearance: The authors have to specify the epidermis area analyzed in the Fig2 E and F.

While we do not notice any regional differences in Merkel cell appearance in the dorsal skin, we have shown images from similar regions of the dorsal epidermis in all our figures.

- The data proposed to show that the number of MC cluster is increased in Ezh1/2 DKO compared to WT is not relevant (supp Fig2F). How do the authors define MC cluster? If they are refereeing to touch dome (TD), they need to present a staining showing TD of the IFE (CD200, see Woo, 2010, Development) and show an increase in the number of TD stained and observed. Pictures illustrating supplemental Fig2 E and F are missing (touch dome quantification).

As we discussed above in response to comments from reviewer 1, we attempted to perform CD200 staining multiple times. We contacted Dr. Owens (the corresponding author from Woo, 2010) and obtained information on the antibodies and protocol used, but we were unable to replicate the data.
Lacking a definitive TD marker, we defined a "MC cluster" as a group of >3 K18+ Merkel cells in which no two cells are located more than 30μm from each other. Please note that touch domes are defined as Merkel cell clusters located in and around guard hairs. In 2KO skin, however, Merkel cell clusters appear around different hair types as well as in the interfollicular epidermis. We thus called them Merkel cell clusters and not touch domes. This explanation is now mentioned in the main text of the manuscript.
Pictures illustrating quantifications in supplemental Fig2 E and F (now SFigure2 I) are provided in Figure 1 A and B.

- *The data for the whisker follicles need to be presented.*

We analyzed proliferation of Merkel cells in both whisker and paw regions and found that K20+ cells are non-proliferative in both 2KO and WT mice. Due to the embedding protocol used at the time that Ezh1/2 2KO embryos were collected, we were unable to obtain sections showing paw or whisker regions for this analysis. However, Merkel cells found in E16 back skin were non-proliferative, consistent with data found for P0 back skin, and thus we don't expect any differences between these regions.

Figure3:
- *What is the proportion of cells that are committed toward MC lineage in Ezh1/2 DKO?*

We performed quantification of the number of K5+ epidermal progenitor cells in WT and 2KO back skin and did not observe any differences. Without an early Merkel cell marker or a progenitor cell marker to use, we cannot uncover whether the number of cells committed toward Merkel cell lineage changes in Ezh1/2 2KO skin.

- *In Fig 3E-F and supp Fig3, the author suggest that the keratinocyte Sox2+ observed will become MC. This statement should be accompanied by a quantification of the number of Sox2+K20- at from E15 to P0 to show that this population decreases, comforting the idea that Sox2 is an early marker of MC.*

We have provided a detailed analysis of Sox2+/-K20+/- populations from E15 to P0 to substantiate our original claim (Figure 3E). Our quantifications showed that indeed the Sox2+K20- population decreases while the Sox2+K20+ population increases over time.

- *Is Sox2 expression localized sporadically in the IFE or is it located in MC cluster (TD)?*

We have not observed a sporadic presence of Sox2+ cells in the interfollicular epidermis. All Sox2+ cells were located in Merkel cell clusters.

Figure 4:
- *The authors do not specify the area of the skin where the MC quantification has been performed. The whisker follicles and paw epidermis should be quantified.*

Merkel cell quantifications were presented in the original paper and were performed on dorsal skin epidermis. Whisker follicles and paw quantifications in WT/Sox2cKO skin are now included in the current submission (SFigure4A-E). We also included in figure legends the description of total length analyzed for each quantification. We also provide complete information on total number of Merkel cells counted for each staining in the Supplementary Table 2.

- *In Sox2 loss of function, the number of MC scored and the length of epidermis quantified have to be specified.*

We have included information on the number of Merkel cells scored in the Supplementary Table 2 and the length quantified in the figure legend.

- In Sox2 loss of function, there is a significant difference in the cells positive for K18 and K20. Can the author comment on that?

We performed statistical analysis of K18+ (Figure4C) and K20+ (Figure4D) cells in Sox2cKO and observed statistical to be not significant (p=0.1384). Any differences are minor and likely reflect antibody efficiency.

- No quantification of the Rab3C + cells in Sox2 loss of function (Supp Fig4).

We have removed this image and now present EM data of Sox2cKO skins and quantifications (Figure 4E). The quantification of Rab3C+ cells was present in Figure 5C of the original submission, which has been moved to Figure 6C for the current version.

- Active caspase 3 immunostaining has been performed at P2. If the MC are not specified because of an increase of cell death, the authors should assess activated caspase3 much more earlier (around the moment of MC specification: E16, E17, E18). Moreover, the authors have to take into account the possibility that the MC progenitors could die. Besides, there is not positive control for caspase 3 staining.

As discussed above, in response to comments from reviewer 1, we first analyzed when fully differentiated Merkel cells appear in WT skin and found that first K20+ cells appear at E17 (Figure 2F and 3E). We next analyzed WT/Sox2cKO skin at E15 and E16, ages that should cover the point prior to specification of Merkel cells, and did not detect enhanced apoptosis in the skin of Sox2cKO mice (SFigure 4K-L). We performed similar analysis of whisker and paw regions and also did not observe an increase of cell death in Sox2-null mice (SFigure 4N-S). The appearance of rare apoptotic cell in WT skin was observed and served as a positive control for activated-caspase 3 IF staining.

- There is no quantification for the number of MC in Sox2 loss of function during adulthood (supp Fig4).

We moved adult WT and Sox2cKO images to Figure 4F and provided quantifications.

- Fig4F and supp Fig4L: the authors should specify if the qPCR data presented have been performed on total skin or sorted cells, the cell isolation protocol, and the eventual gating used to sort the cells.

These figures have been moved to Figure 5A and SFigure 5C. QPCR data presented were performed on total skin, which has been clarified in the figure legends. We could not perform this analysis on sorted Merkel cells because of the low probability of obtaining Sox2cKO Atoh1-GFP pups combined with the low number of Atoh1-GFP+ cells in Sox2cKO mice. From one Atoh1-GFP+ WT pup we can purify 1000 cells, whereas from one Sox2cKO Atoh1-GFP pup we could isolate only 300 cells, severely limiting the ability to obtain enough starting material for this analysis

- Fig4G: what combination of antibodies do the authors use to gate the basal epidermis?

Cell preparation was performed as described in Haeberle H et al PNAS 2004. Gating was done on live Atoh1-GFP+ cells. No antibody staining was performed.

- Math1-GFP is only partially decreased in Sox2cKO mice suggesting that other mechanisms beside Sox2 control Math1 expression and MC specification. This point should be clearly discussed.

This point was also addressed above in the comments for Reviewer 1, point 5. We analyzed the number of Atoh1-GFP+ cells at P0 Sox2cKO and observed 3-fold reduction compared to WT. Interestingly, at P35 there was a complete absence of Atoh1-GFP+ cells in Sox2cKO skin, whereas these cells were present in WT (Figure 5G). Thus, sustained expression of Atoh1-GFP is dependent on Sox2. We speculate that during the Merkel cell specification the loss of Polycomb repression leads to initial Atoh1 expression, consistent with previously published data showing that Atoh1 is a Polycomb target gene (Lien et al Cell Stem Cell 2011 and Ezhkova et al G&D 2011). However, we speculate that Atoh1 expression cannot be sustained or promoted without the function of Sox2

transcription factor. Additionally, the Atoh1-GFP fusion may have a lower turnover rate than endogenous Atoh1 resulting in the presence of a few Atoh1-GFP+ cells in Sox2cKO skin at P0. We addressed this point in the discussion section of the main text.

Figure 5:
- Fig5A right panel: the quantification of the MC number in Sox2/Ezh1/2 TKO mice is missing.

The quantification for Merkel cell number in the Ezh1/2 Sox2 3KO mice was provided for each marker used and is included in the Supplementary Table 2.

- supp Fig5G: there is a discrepancy in the number of MC observed depending on the marker used to detect them (K18, K20, Rab3c). The authors need to present the number of MC quantified and the length of epidermis quantified for each time point. They should also demonstrate the statistical relevance of their data for each condition.

We now present the number of Merkel cells quantified in the Supplementary Table 2 and the length of epidermis quantified for each time point in the figure legend.
We also performed the statistical analysis of the data for each condition and found that the number of K18+ cells in 3KO skin is significantly higher than for K20+ or Rab3c+ cells. Based on the published data as well as our own studies, K20 and Rab3c are markers of fully differentiated Merkel cells. Our IF data for K20 (Figure 5D) and Rab3c (Figure 5E) are consistent with our EM data (Figure 5C) and show a drastic decrease in the number of fully differentiated Merkel cells in 3KO mice. It is possible that loss of Ezh1/2 leads to activation of the *keratin 18* gene that is less dependent on Sox2 function. This is consistent with the fact that keratin 18 gene is a direct target of Polycomb repression (Lien et al Cell Stem Cell 2011) and observed upregulation of K18 in Ezh1/2 2KO basal cells vs WT.

- Supp Fig5H and I: the whiskers IFE is analyzed but not the follicle. What about the number of MC in Sox2/Ezh1/2 TKO whisker follicles and paw epidermis?

We have now provided images and quantifications for whisker follicles and paw epidermis (SFigure 5H-L). These data clearly show that the number of Merkel cells decreases for all regions in 3KO mice compared to WT.

Referee #3

This study investigates the important subject of the role of the key epigenetic regulator, the polycomb complex, in tissue stem cells, using the epidermis as a model. The introduction is clear and concise, but a summary of the EZH1/2 knockout epidermal stem cell and hair follicle phenotypes reported previously and a cartoon to show the how the Merkel cell (MC) lineage is related to other epidermal lineages might help a general reader.

Fig 1 presents clear evidence that EZH1/2 null epidermis has increased numbers of MC, positive for markers and by EM. Functional analysis has not been performed, but as the focus of the paper is on lineage determination this is perhaps not essential.

In Fig 2 the H3K27me3 mark, absent from mutant keratinocytes, is also found to be missing in MC, consistent with MC being keratinocyte lineage derived. A lineage tracing experiment with a histone-GFP transgene expressed from a Krt14 promoter confirms the common origin of MC and keratinocytes and cell cycle staining demonstrates that MC are post mitotic in wild type and null epidermis, so the increased number of MC is not due to a failure of cycle exit. Interestingly MC appear earlier in null than in wild type epidermis (E16 vs 17). The increase in the number of MC clusters in null skin supports the hypothesis that the mutation affects lineage determination.

Fig 3 examines differential gene expression comparing genes expressed in MC in wild type and null epidermis. Sox2 emerges as a highly differentially expressed transcript. That Sox2 is directly regulated by PRC is confirmed by the presence of H3K27me3 marks at the Sox2 gene. Sox2 is expressed in MC and appears in embryonic skin just prior to the first MC.

Fig 4 demonstrates that MC are depleted in Sox2 null epidermis. This phenotype is associated with a decrease in expression of Atoh1, an essential determinant of MC differentiation. Sox2 is shown to directly regulate the Atoh1 gene.

In Fig5 An epistasis experiment in EZH1/2 Sox2 triple null mice is performed. These animals are depleted of MC consistent with the author's hypothesis that loss of PRC repression leads to activation of Sox2, which in turn promotes transcription of Atoh1, leading to Merkel cell differentiation

This is a well written and clearly presented paper, where a series of high quality experiments are used to provide robust support for a novel pathway directing MC differentiation. The work will be of interest to stem cell biologists as well as MC and epidermal specialists and is appropriate for publication in the EMBO Journal.

We are grateful to the reviewer for finding our work to be of interest to the EMBO Journal. We have included more detailed discussion of previously published data on Ezh1/2 2KO phenotype in introduction. He/she did not have any specific comments/suggestions.

