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N6-methyladenosine Modification of lncRNA Pvt1 Governs Epidermal Stemness

Xiaoyang Wu, Jimmy Lee, Yuchen Wu, Bryan Harada, Yuanyuan Li, Jing Zhao, Chuan He, and Yanlei Ma
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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 7th Aug 2020

Dear Xiaoyang,

Thank you again for sharing your work and the submission of your manuscript (EMBOJ-2020-106276) to The EMBO Journal. Your manuscript has been sent to three reviewers, and we have received reports from two of them. Please note that while feedback from referee #3 is pending at this stage, we have in light of the other reviewers' input decided to proceed with our decision to ensure an expedited processing.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. In particular, referee #1 raises concerns regarding potentially confounding effects of MYC-regulatory elements within the Pvt1 DNA (ref#1, pt 1). This referee also asks you to explore structural consequences of Pvt1 m6A site deletion (ref#1, pt.2). In addition, the reviewers raise a number of points related to controls, rescue experiments, methods annotation as well as discussion of the findings and context, which would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments, pending there are no technically overriding concerns presented by referee #3.

I will share the comments from referee #3 as soon as we receive them.

Please let me know any time if you have additional questions or need further input on the referee comments.

Please see below for additional instructions for preparing your revised manuscript.

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

Kind regards,

Daniel

Daniel Klimmeck, PhD

Editor
The EMBO Journal

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Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).

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

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also https://www.embopress.org/page/journal/14602075/authorguide#availabilityofpublishedmaterial). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at

https://www.embopress.org/page/journal/14602075/authorguide#referencesformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 5th Nov 2020.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Lee et al.

N6-methyladenosine modification of lncRNA Pvt1 governs epidermal stemness

In this manuscript, the authors have investigated the role of m6A modification on epidermal differentiation. They generated conditional skin-specific METTL14 knock out mice and studied skin morphology and differentiation. The knock out is not lethal and mice are born. However, they are smaller and show a clear skin phenotype. The authors observe aberrant skin development as well as an inhibited wound healing. Using a number of different methods (label-retention, cell proliferation, colony formation, clonal competition and lineage tracing), Lee et al. find that epidermal stemness is impaired in conditional Mettl14 knock out cells. Since METTL14 forms together with METTL3 the catalytic complex that methylates As, the authors investigated dynamic changes of m6A modification of undifferentiated and differentiated epidermal progenitor cells. They find that the lncRNA Pvt1 showed reduced m6A modification upon cell differentiation. The authors identify 5

target sites and a Pvt1 variant with mutations of all five sites (A to G) was not immunoprecipitated with m6A-specific antibodies. Pvt1 was knock out using CRISPR, which led to a similar phenotype as the loss of METTL14. Interestingly, this could be rescued with WT Pvt1 but not with the mutant that does not contain m6A sites. Finally, the authors show that Pvt1 regulates epidermal stemness through myc. It has been shown before that myc, which appears to be located close to the Pvt1 gene on the genome directly interacts with the Pvt1 lncRNA and this interaction regulates myc stability. Finally, the METTL14 phenotype can be rescued by overexpressing myc demonstrating that both proteins function in the same pathway.

This is a clear and well written manuscript reporting relevant and interesting findings. Experiments are solid and sound and the conclusions that are drawn are generally justified. Nevertheless, there are a number of issues that are unclear and further controls are necessary.

1. The authors state that the Pvt1 is very close to the myc locus. Most of the Pvt1 knock out effects on myc could also be explained by deleting a regulatory element localized on the Pvt1 DNA (either sense or antisense) that is important for myc expression. The authors should also look at myc mRNA levels, which should probably not change if the model that Pvt1 stabilizes the protein by direct interaction is correct.

2. Figure 3B: the authors identify five distinct m6A sites. This RNA cannot be immunoprecipitated with anti-m6A antibodies anymore. Although such clean results are somewhat unexpected, the effects are striking. However, it is also possible that 5 A to G mutations may destroy a structural element that would be required for the Pvt1 function. Is there any structure that could be predicted and would change in the mutated variant? The authors could mutate As to Cs as another control.

3. Would myc overexpression also rescue the Pvt1 knock out cells? This should be tested and added to the manuscript.

4. Figure 4A: the FTO experiment could be explained better in the results as well. Is recombinant FTO added? Is there a control that m6A is indeed removed after treatment? Furthermore, the role of FTO appears to be debated. There are publications showing that only the cap-associated m6As are demethylated by this enzyme and others are not really affected. This should be revisited.

5. Page 5, end of first paragraph: the authors state: ...dramatic decrease of global RNA m6A modification....The authors probably mean ...decrease of global mRNA...

6. As further control that METTL3/14 activity and not some other unknown function of METTL14 is the reason for the observed effects, the authors could rescue their knock out cells with a METTL14 mutant that affects complex activity (e.g. Mettl14 D312A, Wang et al. 2016).

7. On page 8 and Figure 3B, the authors characterize the lncRNA Pvt1 and they show relative m6A levels of the mutants of the wt lncRNA. This is most likely an anti-m6A RIP but described neither in the text nor in the Figure legends. This is also true for other experiments that are only sparsely explained. The authors should go through the manuscript again and add more detailed experimental descriptions.

8. Page9 line 2 Figure 3C: They write that the decrease of Pvt1 results in a reduction of MYC protein but the Figure most likely shows qRT-PCR data. Again, this is not described.

9. Page10 Figure 4A: The authors show an immunoprecipitation of Myc and checked the levels of

Pvt1 in FTO knockdown. As control, the authors should show the levels of immunoprecipitated Myc by adding a western blot. Maybe one could also turn the experiment around and pull down Pvt1 and test for Myc binding. This would further strengthen the idea of a direct bind (although this has been reported by another lab before).

Referee #2:

This is an interesting and novel study highlighting the importance of N6-methyladenosine Modification of RNAs in skin stratification and stem cell self-renewal using genetic mouse models and genomic approaches. Remarkably, deletion of a RNA methylase Mettl14 specifically affects methylation of a subset of RNAs, of which several were lncRNAs. Using a candidate approach the authors go on to demonstrate convincingly that it is through the methylation of lncRNA Pvt1, that Mettl14 performs most of its function in skin and epidermal stem cell regulation. Furthermore, banking on the known role on Pvt in regulating Myc, a well-known factor in epidermal stem cells, the authors go on to demonstrate in vivo (in the mouse) via an inducible transgenic system, as well as in vitro, in cultured keratinocytes that Myc is an essential factor that rescues the stem-cell related phenotypes induced by deletion of Mettl14 and Pvt1. The manuscript is very well-written and easy to follow, and the experiments are extremely well-designed and well-executed. The KO models used in the study were appropriate and a rigorous approach provides convincing evidence for the model proposed. The paper is certainly publishable in the current form, but there are a few minor comments that I suggest may be addressed before publication.

1. The authors should more clearly discuss the novelty of their study in the light of known literature.
2. In this paragraph the authors omitted an important paper in the line of evidence of potential stem/progenitor markers, by Sada et al. 2016: "Potential markers for long term epidermal progenitor cells are not clearly defined in vivo, and different models have been proposed for epidermal tissue maintenance (Clayton et al, 2007; Dekoninck et al, 2020; Jones et al, 1995; Lavker & Sun, 1982; Loeffler et al, 1987; Mascre et al, 2012; Mesa et al, 2018; Morris et al, 1985; Potten & Loeffler, 1987; Potten et al, 1982; Rompolas et al, 2016). However, accumulating evidence reveal the existence of distinct basal cell populations with hierarchical organization and proliferation dynamics in skin epidermis, including slow-cycling progenitor cells and committed progenitor cells with limited proliferative potential (Jones et al., 1995; Lavker & Sun, 1982; Loeffler et al., 1987; Mascre et al., 2012; Morris et al., 1985; Potten & Loeffler, 1987; Potten et al., 1982)"
3. There is mistake in the line "2(D) Morphology of primary keratinocytes isolated from WT or MAP4K4 cKO skin." It is holoclones from Mettl14 KO and not MAP4K4 cKO skin (typo).

4. KO/myc is shown in the Suppl. fig 4 but pvt1 inducible KO grafts is mentioned in the Legends

5. In figure 1 it would be helpful to show images of the wound to appreciate the difference in size measured in 1G (ideally at different time points)
6. This statement is confusing: "Mouse Pvt1 gene locates at chromosome 15 with '9 coding exons'. As Pvt1 is a non-coding RNA". If Pvt1 is a non-coding RNA, why does it have 'coding' exons.

Dear Xiaoyang,

Please find enclosed the report of referee #3. This reviewer is more critical overall and raises important issues, which partially overlap with referee #1's comments and should be addressed in the revision.

Please let me know if you have any question related to the referees' points.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

EMBOJ-2020-106276, report referee #3

In their manuscript, Lee et al start by examining the consequences of Mettl14 deletion during epithelial development and in wound healing. They employ a conditional Mettl14 knockout crossed to a constitutive K14-Cre-expressing line (to study epithelial development) or crossed to a tamoxifen-inducible K14-CreERT2 line (to assess wound healing). Deletion of Mettl14 in the skin leads to visible defects in skin development, including altered thickness and altered expression of the stemness marker p63 and the differentiation marker Krt10. Similarly, in the inducible model, wound healing in adult animals appears delayed and disorganized. These phenotypes are attributed to the diminished presence of slow cycling progenitor cells and are further recapitulated with in vitro experiments.
In search of mechanism, the authors then abruptly switch gears, and define the RNA methylome in undifferentiated and differentiated epidermal progenitor cells and identify RNAs with differences in m6A methylation, including lncRNAs. Next, the manuscript focuses on Pvt1, which appears to have 5 potential m6 sites.
To study Pvt1, two model systems are employed: (i) locus deletion approach to abrogate Pvt1 and (ii) lentiviral constructs that overexpress WT or 5xmutant of the m6A sites. The results appear to indicate that Pvt1 is also important for epidermal stemness. Next, the authors explore whether the previously proposed interaction between Pvt1 and Myc is relevant to epithelial differentiation and whether Myc overexpression from an inducible construct can rescue Mettl14 defects.
The questions addressed in this manuscript are very interesting and exciting. However, the manuscript suffers from several major weaknesses in its organization, experimental approaches, correlative conclusions, and results interpretation. Since some of the weaknesses are integral to the manuscript content and organization, I would recommend rejection.

(1) Figure 1&2 outline very interesting in vivo and in vitro phenotypes associated with Mettl14 KO, while Figure 3&4 address a completely separate story related to the relationship between Pvt1 and Myc in cell-based systems. They cannot be combined in one manuscript.
(2) Deletion of the Pvt1 locus (as much as 300 Kb) is not an acceptable approach to study the Pvt1-Myc interaction because the gene body of Pvt1 contains Myc enhancers, which are perturbed by the iCas-induced deletion.

(3) Related to point (2), the authors report changes in Myc protein levels but the effects are almost certainly also seen at the level of Myc RNA (due to loss of enhancers and diminished transcription). Similarly, diminished Pvt1-Myc interaction might be due to decreased Myc levels in the cell.
(4) In Figure 4, the authors observe that Myc overexpression rescues Mettl14 deficiency but this is expected due to the mitogenic effect of Myc overexpression and is most likely Mettl14-independent. The correlative nature of the data should be pointed out.
(5) The important question of why Mettl14 loss results in impaired differentiation and would healing is not answered, only addressed in correlative experiments.

Response to referee's comments:

We are delighted that the reviewers have found our work to be novel and of high interest to the readership of *EMBO J*. Each reviewer has made enormously helpful comments. We've now fully addressed these issues, and in doing so, have substantially improved the manuscript and its impact. We've conducted the various experiments suggested by the reviewers and revised the manuscript accordingly as we delineate below. Major changes to the manuscript (text) have been highlighted at the left margin. We really thank all the reviewers for all of their constructive comments!

Reviewers' Comments:

Referee #1:

In this manuscript, the authors have investigated the role of m6A modification on epidermal differentiation. They generated conditional skin-specific METTL14 knock out mice and studied skin morphology and differentiation. The knock out is not lethal and mice are born. However, they are smaller and show a clear skin phenotype. The authors observe aberrant skin development as well as an inhibited wound healing. Using a number of different methods (label-retention, cell proliferation, colony formation, clonal competition and lineage tracing), Lee et al. find that epidermal stemness is impaired in conditional Mettl14 knock out cells. Since METTL14 forms together with METTL3 the catalytic complex that methylates As, the authors investigated dynamic changes of m6A modification of undifferentiated and differentiated epidermal progenitor cells. They find that the lncRNA Pvt1 showed reduced m6A modification upon cell differentiation. The authors identify 5 target sites and a Pvt1 variant with mutations of all five sites (A to G) was not immunoprecipitated with m6A-specific antibodies. Pvt1 was knock out using CRISPR, which led to a similar phenotype as the loss of METTL14. Interestingly, this could be rescued with WT Pvt1 but not with the mutant that does not contain m6A sites. Finally, the authors show that Pvt1 regulates epidermal stemness through myc. It has been shown before that myc, which appears to be located close to the Pvt1 gene on the genome directly interacts with the Pvt1 lncRNA and this interaction regulates myc stability. Finally, the METTL14 phenotype can be rescued by overexpressing myc demonstrating that both proteins function in the same pathway.

This is a clear and well written manuscript reporting relevant and interesting findings. Experiments are solid and sound and the conclusions that are drawn are generally justified. Nevertheless, there are a number of issues that are unclear and further controls are necessary.

1. The authors state that the Pvt1 is very close to the myc locus. Most of the Pvt1 knock out effects on myc could also be explained by deleting a regulatory element localized on the Pvt1 DNA (either sense or antisense) that is important for myc expression. The authors should also look at myc mRNA levels, which should probably not change if the model that Pvt1 stabilizes the protein by direct interaction is correct.

We thank the reviewer for this suggestion. To address the potential effect on *Pvt1* deletion on *MYC* expression, we have examined *MYC* mRNA level by RT-PCR. Loss of *Pvt1* by CRISPR-mediated deletion does not lead to significant changes in transcription of *MYC* (Supplementary Fig. 4B). Additionally, we have tested knockdown of *Pvt1* expression by siRNA. Consistent with the data of *Pvt1* KO, siRNA-mediated knockdown of *Pvt1* leads to reduced cell proliferation and impaired colony formation efficiency *in vitro* (Supplementary Fig. 4C and D). Together, the new results strongly suggest that the Pvt1 regulates epidermal stemness through interaction and regulation of MYC protein stability, not through changing of *MYC* transcription.

2. Figure 3B: the authors identify five distinct m6A sites. This RNA cannot be immunoprecipitated with anti-m6A antibodies anymore. Although such clean results are somewhat unexpected, the effects are striking. However, it is also possible that 5 A to G mutations may destroy a structural element that would be required for the Pvt1 function. Is there any structure that could be predicted and would change in the mutated variant? The authors could mutate As to Cs as another control.

We thank the reviewer for pointing out this issue. We carried out *in silico* analysis of Pvt1 with RNAfold web tool (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) from the Vienna RNA Websuite (Gruber et al., 2008). The platform predicts RNA secondary structure based on thermodynamic energy of loops and external bases(Zuker and Stiegler, 1981). From the secondary structure prediction, 3 of the 5 potential methylation A sites are paired (A282, A294 and A446), whereas the other 2 (A303, A452) are unpaired (Supplementary Fig. 4E). To examine the potential effect of these pairing, we have prepared a *Pvt1* mutant in which the three corresponding T residues were mutated to C (A5GT3C). This mutant shows reduced MYC binding (Supplementary Fig. 5C) and cannot rescue CFE *in vitro* (Supplementary Fig. 4F), resembling the effect of A5G mutant. The new results suggest that the defects seen in the methylation mutant is not due to potential secondary structural change of Pvt1.

3. Would myc overexpression also rescue the Pvt1 knock out cells? This should be tested and added to the manuscript.

We prepared MYC overexpression line from *Pvt1* KO cells as suggested. Ectopic expression of *MYC* can restore the CFE *in vitro* (Supplementary Fig. 5F), suggesting that MYC is the key downstream effector of Pvt1 in controlling epidermal stemness.

4. Figure 4A: the FTO experiment could be explained better in the results as well. Is recombinant FTO added? Is there a control that m6A is indeed removed after treatment? Furthermore, the role of FTO appears to be debated. There are publications showing that only the cap-associated m6As are demethylated by this enzyme and others are not really affected. This should be revisited.

We used recombinant FTO to demethylate RNA samples, as described before (Wei et al., 2018). RT-PCR analysis shows that Pvt1 can be efficiently demethylated after the treatment *in vitro* (Supplementary Fig. 5B).

5. Page 5, end of first paragraph: the authors state: ...dramatic decrease of global RNA m6A modification....The authors probably mean ...decrease of global mRNA...

Revised as suggested.

6. As further control that METTL3/14 activity and not some other unknown function of METTL14 is the reason for the observed effects, the authors could rescue their knock out cells with a METTL14 mutant that affects complex activity (e.g. Mettl14 D312A, Wang et al. 2016).

As suggested, we generated rescued Mettl14 KO cell line with re-expression of WT *Mettl14* or *Mettl14* R298P mutant, which has reduced methyltransferase activity (Wang et al., 2016). The new results show that WT *Mettl14* but not the R298P mutant can restore CFE of KO cells *in vitro*, strongly suggesting that the role of Mettl14 on epidermal stemness requires its RNA methyltransferase activity (Supplementary Fig. 2D).

7. On page 8 and Figure 3B, the authors characterize the lncRNA Pvt1 and they show relative m6A levels of the mutants of the wt lncRNA. This is most likely an anti-m6A RIP but described neither in the text nor in the

Figure legends. This is also true for other experiments that are only sparsely explained. The authors should go through the manuscript again and add more detailed experimental descriptions.
Revised to include the experimental details as suggested (Page 8, and Materials and Methods, Page 17 and 18).

8. Page9 line 2 Figure 3C: They write that the decrease of Pvt1 results in a reduction of MYC protein but the Figure most likely shows qRT-PCR data. Again, this is not described.
We apologize for this mistake. The right panel is quantification of MYC immunoblots data. We have revised the text and legend as suggested (page 9, and Figure legends 3C).

9. Page10 Figure 4A: The authors show an immunoprecipitation of Myc and checked the levels of Pvt1 in FTO knockdown. As control, the authors should show the levels of immunoprecipitated Myc by adding a western blot. Maybe one could also turn the experiment around and pull down Pvt1 and test for Myc binding. This would further strengthen the idea of a direct bind (although this has been reported by another lab before).
We appreciate this suggestion. As recommended, we have included immunoblots data of MYC in the revised manuscript (Supplementary Fig. 5D).

Referee #2:
This is an interesting and novel study highlighting the importance of N6-methyladenosine Modification of RNAs in skin stratification and stem cell self-renewal using genetic mouse models and genomic approaches. Remarkably, deletion of a RNA methylase Mettl14 specifically affects methylation of a subset of RNAs, of which several were lncRNAs. Using a candidate approach the authors go on to demonstrate convincingly that it is through the methylation of lncRNA Pvt1, that Mettl14 performs most of its function in skin and epidermal stem cell regulation. Furthermore, banking on the known role on Pvt in regulating Myc, a well-known factor in epidermal stem cells, the authors go on to demonstrate in vivo (in the mouse) via an inducible transgenic system, as well as in vitro, in cultured keratinocytes that Myc is an essential factor that rescues the stem-cell related phenotypes induced by deletion of Mettl14 and Pvt1. The manuscript is very well-written and easy to follow, and the experiments are extremely well-designed and well-executed. The KO models used in the study were appropriate and a rigorous approach provides convincing evidence for the model proposed. The paper is certainly publishable in the current form, but there are a few minor comments that I suggest may be addressed before publication.
1. The authors should more clearly discuss the novelty of their study in the light of known literature.
We thank the reviewer for this suggestion. We have revised the discussion in the resubmission to have better discussion on the novelty of our findings (Page 12 and 13).

2. In this paragraph the authors omitted an important paper in the line of evidence of potential stem/progenitor markers, by Sada et al. 2016: "Potential markers for long term epidermal progenitor cells are not clearly defined in vivo, and different models have been proposed for epidermal tissue maintenance (Clayton et al, 2007; Dekoninck et al, 2020; Jones et al, 1995; Lavker & Sun, 1982; Loeffler et al, 1987; Mascre et al, 2012; Mesa et al, 2018; Morris et al, 1985; Potten & Loeffler, 1987; Potten et al, 1982; Rompolas et al, 2016). However, accumulating evidence reveal the existence of distinct basal cell populations with hierarchical organization and proliferation dynamics in skin epidermis, including slow-cycling progenitor cells and committed progenitor cells with limited proliferative potential (Jones et al., 1995; Lavker & Sun, 1982; Loeffler et al., 1987; Mascre et al., 2012; Morris et al., 1985; Potten & Loeffler, 1987; Potten et al., 1982)"

New citation included as suggested.

3. There is mistake in the line "2(D) Morphology of primary keratinocytes isolated from WT or MAP4K4 cKO skin." It is holoclones from Mettl14 KO and not MAP4K4 cKO skin (typo).
Corrected.

4. KO/myc is shown in the Suppl. fig 4 but pvt1 inducible KO grafts is mentioned in the Legends
Corrected.

5. In figure 1 it would be helpful to show images of the wound to appreciate the difference in size measured in 1G (ideally at different time points) 6. This statement is confusing: "Mouse Pvt1 gene locates at chromosome 15 with '9 coding exons'. As Pvt1 is a non-coding RNA". If Pvt1 is a non-coding RNA, why does it have 'coding' exons.
Images of wounds at different time points are included in the revised manuscript (Supplementary Fig. 1F). Changed “9 coding exons” to “9 exons”.

referee #3
In their manuscript, Lee et al start by examining the consequences of Mettl14 deletion during epithelial development and in wound healing. They employ a conditional Mettl14 knockout crossed to a constitutive K14-Cre-expressing line (to study epithelial development) or crossed to a tamoxifen-inducible K14-CreERT2 line (to assess wound healing). Deletion of Mettl14 in the skin leads to visible defects in skin development, including altered thickness and altered expression of the stemness marker p63 and the differentiation marker Krt10. Similarly, in the inducible model, wound healing in adult animals appears delayed and disorganized. These phenotypes are attributed to the diminished presence of slow cycling progenitor cells and are further recapitulated with in vitro experiments.
In search of mechanism, the authors then abruptly switch gears, and define the RNA methylome in undifferentiated and differentiated epidermal progenitor cells and identify RNAs with differences in m6A methylation, including lncRNAs. Next, the manuscript focuses on Pvt1, which appears to have 5 potential m6 sites.
To study Pvt1, two model systems are employed: (i) locus deletion approach to abrogate Pvt1 and (ii) lentiviral constructs that overexpress WT or 5xmutant of the m6A sites. The results appear to indicate that Pvt1 is also important for epidermal stemness. Next, the authors explore whether the previously proposed interaction between Pvt1 and Myc is relevant to epithelial differentiation and whether Myc overexpression from an inducible construct can rescue Mettl14 defects.
The questions addressed in this manuscript are very interesting and exciting. However, the manuscript suffers from several major weaknesses in its organization, experimental approaches, correlative conclusions, and results interpretation. Since some of the weaknesses are integral to the manuscript content and organization, I would recommend rejection.
(1) Figure 1&2 outline very interesting in vivo and in vitro phenotypes associated with Mettl14 KO, while Figure 3&4 address a completely separate story related to the relationship between Pvt1 and Myc in cell-based systems. They cannot be combined in one manuscript.
We are sorry for not making the organization of the manuscript clear in the initial submission. The Figure 3 and 4 are addressing the molecular mechanism whereby Mettl14 complex and m^6A modification regulate epidermal stemness. Our results show that Pvt1 is an important lncRNA with m^6A modification, which is

critical for its interaction with MYC. Loss of Pvt1 modification leads to MYC degradation and impaired epidermal stemness, and most importantly, ectopic expression of MYC can rescue the defects in both Pvt1 KO cells and Mettl14 KO cells, strongly suggesting that the Pvt1/MYC axis is the key downstream pathway of Mettl14 methyltransferase complex in skin epidermal stem cells.
We have also revised the Discussion section in the revised manuscript to highlight the novelty and relevance of our findings (Page 12 and 13).

(2) Deletion of the Pvt1 locus (as much as 300 Kb) is not an acceptable approach to study the Pvt1-Myc interaction because the gene body of Pvt1 contains Myc enhancers, which are perturbed by the iCas-induced deletion.
We thank the reviewer for pointing out this issue, which was also raised by reviewer 1. As discussed above, we have examined *MYC* mRNA level by RT-PCR. Loss of *Pvt1* by CRISPR-mediated deletion (~50% reduction) does not lead to detectible changes in transcription of *MYC* (Supplementary Fig. 4B). Additionally, we have tested knockdown of *Pvt1* expression by siRNA. Consistent with the data of *Pvt1* KO, siRNA-mediated knockdown of *Pvt1* leads to reduced cell proliferation and impaired colony formation efficiency *in vitro* (Supplementary Fig. 4C and D). Together, the new results strongly suggest that the Pvt1 regulates epidermal stemness through interaction and regulation of MYC protein stability, not through changing of *MYC* transcription.

(3) Related to point (2), the authors report changes in Myc protein levels but the effects are almost certainly also seen at the level of Myc RNA (due to loss of enhancers and diminished transcription). Similarly, diminished Pvt1-Myc interaction might be due to decreased Myc levels in the cell.
As discussed above, we have examined *MYC* mRNA level by RT-PCR. For Pvt1-Myc interaction studies, we have included immunoblot results of precipitated MYC to verify that similar amount of MYC in the samples (Supplementary Fig. 5D).

(4) In Figure 4, the authors observe that Myc overexpression rescues Mettl14 deficiency but this is expected due to the mitogenic effect of Myc overexpression and is most likely Mettl14-independent. The correlative nature of the data should be pointed out.
KO of *Mettl14* leads to reduced MYC protein stability and decreased MYC protein level (Fig. 4B and C). Ectopic expression of *MYC* can rescue *Mettl14* KO phenotypes *in vitro* and in organotypic culture and skin transplantation models, providing compelling evidence that Mettl14 regulates epidermal stemness via the Pvt1-MYC pathway. However, we thank the reviewer for pointing out the potential caveat, and we have included new discussion in the revised manuscript to discuss this issue (Page 13).

(5) The important question of why Mettl14 loss results in impaired differentiation and would healing is not answered, only addressed in correlative experiments.
As discussed above (point 1), the Figure 3 and 4 are addressing the molecular mechanism whereby Mettl14 complex and m^6A modification regulate epidermal stemness. Our results strongly suggest that the Pvt1/MYC axis is the key downstream pathway of Mettl14 methyltransferase complex in skin epidermal stem cells.

References:

Gruber, A.R., Lorenz, R., Bernhart, S.H., Neuböck, R., and Hofacker, I.L. (2008). The Vienna RNA websuite. Nucleic Acids Res *36*, W70-74.

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1st Revision - Editorial Decision 22nd Jan 2021

Dear Xiaoyang,

Thank you for submitting your revised manuscript (EMBOJ-2020-106276R) to The EMBO Journal. Please accept my sincere apologies for getting back to you with this unusual protraction due to delayed reviewer input during re-review as well as detailed discussions here in the team. Your amended study was sent back to the three referees, and we have received comments from all of them, which I enclose below.

While referee #3 remains overall critical, the other referees stated that the raised issues have been comprehensively resolved and they 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.

Please consider the remaining points by reviewer #3 as follows:
>>Clarify technical and biological replicates for the experiment depicted in Suppl. Fig. 4B.
>>Revisit annotation of experimental conditions shown in Suppl. Fig 5D and complement the figure legend.

Also, we need you to take care of a number of points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might remember from your previous work, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal.
I look forward to your adjusted manuscript files.

Again, we are happy to swiftly move forward with acceptance of this work upon re-submission.
Please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Introduce ORCID IDs for all corresponding authors (Y.M.) via our online manuscript system. Please see below for additional information.

>> Please specify distinct author contributions for Y.L. .

>> Recheck callouts and their correct order in the main text for figures 3H and EV6.

>> Provide figures as individual, high-resolution .tiff files. The figure legends should stay in the manuscript.

>> Please enter the following funding information for your study into our online system: T32, CA 9594-30.

>>Avoid textual redundance with your 2020 study in the introduction, results and discussion parts (PMID: 32239614).

>> Dataset EV legends: Please add the legend to the respective excel table in a new table and correct the nomenclature to "Dataset EV1" and "Dataset EV2".

>>Share the sequencing data on GEO or similar database as freely accessible entry and update the 'Data accessibility' section as well as the Author Checklist accordingly.

>> Add a ToC on the first page of the Appendix; Remove legends to spreadsheets, and correct nomenclature to "Appendix Figure S1" etc. in the main text and legends.

>> In line with the policies of our journal, we kindly ask you to provide uncropped source data for Suppl. Fig 5G.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
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Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 22nd Apr 2021.

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Referee #1:

In their revised manuscript, Lee et al. have addressed all points that I had raised on their previous version. Additional and experiments and controls have been performed and they responded adequately. Therefore, I am satisfied with the revised manuscript.

Referee #2:

The authors appropriately addressed all my concerns, and in my opinion, all the other concerns raised the two other reviewers. Therefore I believe the manuscript is now publishable in the current form.

Referee #3:

The manuscript does not appear to have been improved significantly. Even superficially, the abstract and figures from the initial and revised version appear identical, indicating that the authors made little effort to address the reviewers's comments.
In addition, none of the reviewer's comments were addressed. As examples, when criticizing the "organization" of the manuscript, the reviewer was referring to the fact that this manuscript is stitched from two independent model systems and topics (Point 1). This was not addressed in the revised version or in response to reviewers. Point 2 was also not addressed. Suppl. Figure 4D is not convincing at all and it appears to be from technical rather than biological replicates. On a related note, many of the figures lack statistical analysis. In addressing Point 3, Suppl. Figure 5G is also not convincing. It looks like the IB for MYC suffers from significant artifacts. Finally, the discussion does not sufficiently address the correlative nature of the conclusions. The reviewer recommends

rejection.

Response to referee's comments:

We were delighted that the reviewers found our revision has significantly improved the manuscript. One reviewer had additional comments on the study. We've now addressed these remaining issues as suggested. We really thank the editor and all the reviewers for the constructive comments!

Reviewers' Comments:

Referee #3:

1. Clarify technical and biological replicates for the experiment depicted in Suppl. Fig. 4B.

Biological replicates were performed for results in Suppl. Fig. 4B. We have revised the Figure legend to include this information.

2. Revisit annotation of experimental conditions shown in Suppl. Fig 5D and complement the figure legend.

We have revisited and confirmed the information shown in Suppl. Fig. 5D and legend.

2nd Revision - Editorial Decision 15th Feb 2021

Dear Xiaoyang,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

Also in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work.
Please see the following link for a representative example:
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel

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Corresponding Author Name: Xiaoyang Wu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-106276

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen based on our preliminary test and prior experience with the same type of experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size for animal experiments was chosen based on preliminary examination of the phenotype, and our previous experience with the same type of experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There is no sample exclusion for all the in vitro analysis. For in vivo experiments, animals that died before the end of the experiment were excluded. The exclusion criteria is pre-established.
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.	No randomization or blinding was used in this study.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization or blinding was used in this study.
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.	No blinding was used in this study.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was used in this study.
5. For every figure, are statistical tests justified as appropriate?	All statistical tests are justified.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We have used multiple statistical tools to test the signifcance, as described in the manuscript.
Is there an estimate of variation within each group of data?	No.

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Is the variance similar between the groups that are being statistically compared?	Yes.

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).	We have included the inforamtion for all the antibodies in the Material and Methods section in the revised manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used only primary mouse keratinocytes in this study. Potential contamination with mycoplasma was screened using the ATCC universal mycoplasma detection kit.

* 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.	All the mice were bred and maintained in the ARC (animal resource center) of the University of Chicago in accordance with institutional guidelines. Strain, gender, and other informations have been included in the Method & Material sections in the revised manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments with mice have been approved by IACUC (Institutional Animal Care and use Committee) of the University of Chicago.
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.	It is compliant with the guideline.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subjects involved.
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.	No human subjects involved.
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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	We will deposit the sequencing data following the guideline.
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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.	No.