EMBO reports

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# A non-canonical function of Ezh2 preserves immune homeostasis

Ajithkumar Vasanthakumar, Dakang Xu, Aaron Lun, Andrew J. Kueh, Klaas P.J.M. van Gisbergen, Nadia Iannarella, Xiaofang Li, Liang Yu, Die Wang, Bryan R.G. Williams, Stanley C. W. Lee, Ian J. Majewski, Dale I. Godfrey, Gordon K. Smyth, Warren S. Alexander, Marco J. Herold, Axel Kallies, Stephen L. Nutt, and Rhys S. Allan

*Corresponding authors: Stephen L. Nutt and Rhys S. Allan, The Walter and Eliza Hall Institute*

**Review timeline:**

| | |
|---|---|
| Submission date: | 19 August 2016 |
| Editorial decision: | 13 September 2016 |
| Revision received: | 18 December 2016 |
| Editorial decision: | 16 January 2017 |
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Editor: Achim Breiling

## Transaction Report:

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

1st Editorial Decision 13 September 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees acknowledge the potential interest of the findings and support the publication of the manuscript after revision. All three referees have raised a number of concerns and have come up with suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which we feel need all to be addressed during the revision. As the reports are below, I will not detail them here.

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

REFEREE REPORTS
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Referee #1:

The manuscript A non-canonical function for Ezh2 in the preservation of immune homeostasis submitted by Vasanthakumar et al describes a non-canonical role for the histone methyltransferase Ezh2 in the development of NKT-cells. The authors provide data to suggest that inactivation of Ezh2 leads to decreased protein degradation of the critical NKT regulator PLZF, resulting in perturbed T-cell development. They further provide biochemical evidence for an EZH2-mediated methyl degron in PLZF at lysine residue K430.
Overall, the experiments are well designed, well controlled and credible. While the concept of non-histone Ezh2 targets is not entirely novel, PLZF has not been described as an Ezh2 target and the physiologic relevance of Ezh2-mediated PLZF protein turnover is well characterized here.
Novelty of the manuscript is in part limited by a prior manuscript from Tarakhovsky's group (which the authors appropriately reference). The mechanistic novelty described here in my view compensates for this and the findings described here are of interest to a broader audience and fit well into the journal.
I have a number of minor and mostly technical critiques as follows:

1.) Figure 1 Flow plots:

General comment - did the authors confirm complete excision of floxed sequences?

- 1a) There is no major effect on CD4/8 double positive cell development using the CD4-Cre strain. It might be worth pointing out that Ezh2/PRC2 has a very strong effect if deletion is accomplished using Mx-Cre (presumably leading to an earlier differentiation block) (Genes Dev. 2012 Apr 1;26(7):651-6.).
- 1d) It is curious that the H3K27me3 mark goes up in the Ezh2-ko. While it is true that Ezh1 can to some degree compensate, Ezh1 is thought to be a weak methyl-transferase (Mol Cell. 2008 Nov 21;32(4):503-18.) and Ezh2 in many cell types appears to be the major methyl-transferase (Mol Cell. 2008 Nov 21;32(4):491-502.; Proc Natl Acad Sci U S A. 2012 Mar 27;109(13):5028-33.). Are the authors sure that this is reproducible and there is no legend mix-up?

2.) Figure 4d - There is no size marker, and no PLZF ab was used. Are the authors sure this band corresponds to PLZF?

3.) Figure 5c - the fonts used to label Cd1d-/- and Ezh2cKO are mismatched. Figure 5d) the far right bar (gray) appears to be mislabeled as Ezh2cko but ?should be labelled as ?Ezh2cko/Cd1d-/-?

4.) Figure S2c is undecipherable and needs editing. The information contained is presumably relevant.

5.) Figure S3b shows an only minuscule effect on the stage subsets. How does this reconcile with the more pronounced differences in figure 2a)?

6.) The text on page 10/11 seems to reference the incorrect panels of Figure S3.

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Referee #2:

Ezh2 acts primarily as a gene silencer, but emerging evidence indicates that Ezh2 can also act as a transcription activator. Authors demonstrate a chromatin-independent role for Ezh2 in the control of T cell developmental processes and immune homeostasis. They found that specific deletion of T cells during DP-stage of thymic development led to a pronounced expansion of NKT cells. Authors suggest that the effect of Ezh2 on repressing NKT cell development is regulated by a chromatin-independent function of Ezh2, because removal of Suz12 or Eed, which form complex with Ezh2 to silence gene programs. This is because that Ezh2 directly methylated PLZF, the lineage defining transcription factors for NKT cells, leading to its ubiquitination and subsequent degradation. Sustained PLZF expression in Ezh2-defiicent mice was associated with the expansion of a subset of NKT cells that cause immune perturbation. These data are interesting as they illuminate a new function of Ezh2 in regulating protein stability and function. To substantially support their

 

conclusion, authors may need to clarify the following concerns.

The major concerns are:

1. Fig.1d showed that there was no reduction of H3K27me3 in Ezh2cko NKT cells when compared to WT NKT cells. This analysis was done using flow cytometry staining. Would authors be able to examine the amount of H3K27me3 using Western blot to validate this finding?

2. In Fig.1, it is unclear if deletion of Ezh2 might lead to destabilization of PRC2 and subsequent reduction of Eed and Suz12 in NKT cells. In addition, if Ezh1 plays an important role to compensate Ezh2 in the stabilization of PRC2, could Ezh1 form the complex with Suz12 and Eed in NKT cells lacking Ezh2? Since Fig.1d shows that Ezh1 was increased in Ezh2cko NKT cells, could it be possible that increased expression of Ezh1 may enhance the expansion of NKT cells in cooperation with PRC2 components Szu12 and Eed? Addressing these questions will be important for authors to establish their hypothesis that Ezh2 has PRC2-mediated chromatin-independent effects in NKT development. One may argue that increased expression of Ezh2 might contribute to the expansion of NKT cells, although it may be unlikely.

3. Specific inhibitors for reducing H3K27me3 are available. For example, GSK126 selectively reduces Ezh2-mediated H3K27me3, whereas UNC1999 is able to decrease H3K27me3 catalyzed by both Ezh2 and Ezh1. Neither compound interferes with the expression of Ezh2 protein. In vivo administration of these compounds would allow authors to provide more evidence if PRC2-indepepdent function of Ezh2 may influence NKT cell development.

4. Fig.3e, RNAseq track showing read mapping to the Zbtb16 gene in WT and Ezh2cKO from stage 1-3 NKT cells. Could authors use CHIP analysis to test if Ezh2 binds to the promoter region of Zbtb16 locus?

5. In Fig.4c, authors show that the presence of Ezh2 led to increased PLZF methylation. Given the role of Ezh2 SET domain (which is deleted in Ezh2cko) in the methylation of histone protein, will this Ezh2 SET domain be required for mediating methylation of PLZF? Will this effect of Ezh2 methylation of PLZF require the presence of SUZ12 and / or EED? Furthermore, will knockout of EZH1 lead to altered stability of PLZF?

6. Data shown in Fig.S3b suggest that introduction of K430R PLZF led to an increased proportion of NKT cells at stage 1 and a corresponding decrease in stage 3. However, in Fig.2a, authors show that loss of Ezh2 led to increase stage 2 NKT cells without significant changes in Stage 1 cell population. Give the role of Ezh2 in destabilizing PLZF, one may expect that introduction of PLZF causes an increased fraction of Stage 2 cells. A flow cytometic analysis will be necessary to demonstrate that whether PLZF is highly induced in the stage 2 NKT cells from Ezh2cko mice.

7. Finally, authors conclude that they identified a role for chromatin-independent functions of Ezh2. The term of chromatin-independent function seems an ambiguous. Since Ezh2, Suz12 and Eed form PRC2 to catalyze H3K27me3, it seems that an appropriate conclusion could be relevant to PRC2-independent function of Ezh2 in this study. To further refine the conclusion, authors may need to consider additional supportive evidence, such as: 1) whether loss of Ezh2 in NKT cells affects the expression of Suz12 and Eed; and 2) whether loss of Suz12 or Eed in NKT cells affects the expression of Ezh2.

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Referee #3:

The paper by Vasanthakumar et al describes an expansion of natural killer T (NKT) cells and subsequent alteration of the immune homeostasis in mice conditionally mutant in their T cells for PRC2 component Ezh2 (deleted exon encoding lysine-methyltransferase SET domain).

The authors show an expansion of NKT cells which appears unrelated to the methylation of lysine 27 in histone H3, the well known activity of Ezh2. On the other hand, the mutant NKT population is enriched in cells that express high levels of DNA-binding protein PLZF, a transcription factor

 

essential for NKT development. In the absence of changes in mRNA encoding PLZF, the authors examine whether decreased protein destabilization accounts for PLZF protein accumulation in mutant cells. The possibility that mutant Ezh2 fails to generate a methyl-degron on PLZF that would escape proteolysis is then assessed in a 293T cell system. There, the authors show an interaction between Ezh2 and PLZF, polyubiquitylation of wild type PLZF very much decreased when a candidate lysine residue in PLZF is turned into a non methylatable arginine, and stabilization of PLZF upon downregulation (shRNA) of Ezh2. All of which sustains a hypothesis by which regulation of PLZF levels through the a Ezh2-dependent methyl degron has been cancelled in cells expressing a methylation-incompetent form of Ezh2.

The in vivo approach to test this hypothesis was not conclusive, though. The attempted experiment (retroviral transduction of wild type and mutant PLZF), however, would have interrogated PLZF protein levels rather than Ezh2-dependent modification of PLZF in these cells. A rather direct approach, analyzing PLZF in mutant cells or in cells derived from mutant mice would have been preferred. It is possible that cell numbers are of no help and that is a reason for the authors to have skipped such an approach, but this should have been discussed. Certainly, were the experiment to be technically feasible it should be attempted.

Despite this caveat, the findings are important in exposing regulatory functions of Polycomb products beyond the usual interest on chromatin modification(s).

Minor:

It is a bit surprising that reporting a non-histone methylation function for Ezh2 there is hardly any discussion about it or its relation to other protein methylation events.

While for the authors it may be straightforward that PLZF is the product of the Zbtb16 locus, it would be better if this was indicated somewhere in the text describing results in Fig. 3.

References by Smyth GK 2004 or Xie et al. are incomplete.

It is the authors' manuscript but the closing paragraph in the discussion relates little to the core of the paper and does not add much in general.

1st Revision - authors' response 18 December 2016

*We thank the referees for their time and constructive comments. Our responses (in italics) are included below.*

Referee #1:

Overall, the experiments are well designed, well controlled and credible. While the concept of non-histone Ezh2 targets is not entirely novel, PLZF has not been described as an Ezh2 target and the physiologic relevance of Ezh2-mediated PLZF protein turnover is well characterized here.

I have a number of minor and mostly technical critiques as follows:

1.) Figure 1 Flow plots. General comment - did the authors confirm complete excision of floxed sequences?

*Yes, we have confirmed the excision of the floxed sequences in $CD4^+CD8^+$ thymocytes from Ezh2, Suz12 and $Eed^{cKO}$ mice by PCR (please see Figure EV1).*

1a) There is no major effect on CD4/8 double positive cell development using the CD4-Cre strain. It might be worth pointing out that Ezh2/PRC2 has a very strong effect if deletion is accomplished using Mx-Cre (presumably leading to an earlier differentiation block) (Genes Dev. 2012 Apr 1;26(7):651-6.).

*We have now included this information on page 3 of the manuscript.*

 

1d) It is curious that the H3K27me3 mark goes up in the Ezh2-ko. While it is true that Ezh1 can to some degree compensate, Ezh1 is thought to be a weak methyl-transferase (Mol Cell. 2008 Nov 21;32(4):503-18.) and Ezh2 in many cell types appears to be the major methyl-transferase (Mol Cell. 2008 Nov 21;32(4):491-502.; Proc Natl Acad Sci U S A. 2012 Mar 27;109(13):5028-33.). Are the authors sure that this is reproducible and there is no legend mix-up?

*We have now confirmed this result by western blot of NKT cells isolated from 5 individual Ezh2$^{cKO}$ mice to reveal that a majority of H3K27me3 remains in these cells (Figure 1e).*

2.) Figure 4d - There is no size marker, and no PLZF ab was used. Are the authors sure this band corresponds to PLZF?

*We apologize to the reviewer if this was unclear. In this case we co-expressed Flag-tagged PLZF with ubiquitin-HA and then pulled down PLZF with anti-Flag antibodies. We then assessed the presence of ubiquitin by blotting for HA. We did examine the input PLZF by immunoblot with PLZF-specific antibody in the "input". We have now added (Flag) in superscript in the figure to make this clearer.*

3.) Figure 5c - the fonts used to label Cd1d-/- and Ezh2cKO are mismatched. Figure 5d) the far right bar (gray) appears to be mislabeled as Ezh2cko but should be labelled as ?Ezh2cko/Cd1d-/-?

*We have now fixed this error.*

4.) Figure S2c is undecipherable and needs editing. The information contained is presumably relevant.

*This figure has been removed as it is more clearly articulated in the text.*

5.) Figure S3b shows an only minuscule effect on the stage subsets. How does this reconcile with the more pronounced differences in figure 2a)?

*As we surmised in the discussion these are technically challenging experiments with a range of variables. Nevertheless, we observed a skewing of the cells transduced with the K430R mutant towards stage 1 and a corresponding drop in the proportion of stage 3 NKT cells. This suggests that stabilizing PLZF does indeed alter NKT cell development, however, the modest effects observed also imply that this mechanism may not be the only one operating in the expanded NKT cells from the Ezh2$^{cKO}$ mice. We speculate the Ezh2-PLZF methyl degron is required at the transition between stages 1 and 2 to downregulate PLZF protein prior to transcriptional repression of Zbtb16 at stage 3, and that introducing this stabilized version of PLZF into early NKT cell progenitors (stage 0) may lead to the early block at stage 1.*

6.) The text on page 10/11 seems to reference the incorrect panels of Figure S3.

*This has now been altered.*

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Referee #2:

These data are interesting as they illuminate a new function of Ezh2 in regulating protein stability and function. To substantially support their conclusion, authors may need to clarify the following concerns.

<u>The major concerns are:</u>

1. Fig.1d showed that there was no reduction of H3K27me3 in Ezh2cko NKT cells when compared to WT NKT cells. This analysis was done using flow cytometry staining. Would authors be able to examine the amount of H3K27me3 using Western blot to validate this finding?

 

*We have performed western blot for H3K27me3 on NKT cells from five individual Ezh2$^{cKO}$ mice (now included in Figure 1e), which indeed validates our flow cytometry staining.*

2. In Fig.1, it is unclear if deletion of Ezh2 might lead to destabilization of PRC2 and subsequent reduction of Eed and Suz12 in NKT cells.

*We have now examined the levels of Ezh2 in thymocytes from the Suz12 and Eed$^{cKO}$ mice to show that Ezh2 is substantially reduced suggesting that PRC2 stability is lost when these core factors are removed (Figure EV2b). In addition in Figure EV2c we have shown that Suz12 levels are not altered in thymocytes from Ezh2$^{cKO}$ mice, indicating that loss of Ezh2 itself does not destabilize PRC2.*

In addition, if Ezh1 plays an important role to compensate Ezh2 in the stabilization of PRC2, could Ezh1 form the complex with Suz12 and Eed in NKT cells lacking Ezh2?

*Yes, while we have not shown this in our study this phenomenon has been demonstrated a number of groups (namely Shen et al., Mol Cell 2008 and Xie et al., Cell Stem Cell 2013).*

Since Fig.1d shows that Ezh1 was increased in Ezh2cko NKT cells, could it be possible that increased expression of Ezh1 may enhance the expansion of NKT cells in cooperation with PRC2 components Szu12 and Eed?

*As the reviewer suggests in the context of the current literature, this is an unlikely scenario.*

3. Specific inhibitors for reducing H3K27me3 are available. For example, GSK126 selectively reduces Ezh2-mediated H3K27me3, whereas UNC1999 is able to decrease H3K27me3 catalyzed by both Ezh2 and Ezh1. Neither compound interferes with the expression of Ezh2 protein. In vivo administration of these compounds would allow authors to provide more evidence if PRC2-indepepdent function of Ezh2 may influence NKT cell development.

*This is an interesting suggestion, however, our concern is the use of inhibitors in vivo may have effects on many other cells, which would make such an experiment difficult to interpret. Moreover, establishing the optimal conditions for these experiments would also be very time consuming and we believe this is beyond the scope of the study.*

4. Fig.3e, RNAseq track showing read mapping to the Zbtb16 gene in WT and Ezh2cKO from stage 1-3 NKT cells. Could authors use CHIP analysis to test if Ezh2 binds to the promoter region of Zbtb16 locus?

*This is not a trivial experiment as NKT cells are a rare population and a high number of cells are required to perform ChIP for chromatin binding proteins. Moreover we see very low H3K27me3 levels in the Zbtb16 promoter and no change in Zbtb16 transcription making the presence of Ezh2 in this region irrelevant.*

5. In Fig.4c, authors show that the presence of Ezh2 led to increased PLZF methylation. Given the role of Ezh2 SET domain (which is deleted in Ezh2cko) in the methylation of histone protein, will this Ezh2 SET domain be required for mediating methylation of PLZF?

*We have now added new data showing that inhibiting the enzymatic activity of Ezh2 with a small molecule inhibitor results in a reduction of PLZF methylation suggesting that the Ezh2 SET domain is required for this process (see Figure 4d).*

Will this effect of Ezh2 methylation of PLZF require the presence of SUZ12 and / or EED?

*We have produced Suz12 deficient 293T cells by Crispr/Cas9 to answer this question (Fig. EV4d) and have found that Suz12 does not appear to be required for Ezh2 to methylate PLZF in our system (Fig. EV4e).*

6. Data shown in Fig.S3b suggest that introduction of K430R PLZF led to an increased proportion of NKT cells at stage 1 and a corresponding decrease in stage 3. However, in Fig.2a, authors show that loss of Ezh2 led to increase stage 2 NKT cells without significant changes in Stage 1 cell

 

population. Give the role of Ezh2 in destabilizing PLZF, one may expect that introduction of PLZF causes an increased fraction of Stage 2 cells. A flow cytometic analysis will be necessary to demonstrate that whether PLZF is highly induced in the stage 2 NKT cells from Ezh2cko mice.

*Figure 3c indeed shows that PLZF protein is maintained at high levels in stage 2 NKT cells from the Ezh2$^{cKO}$ mice.*

7. Finally, authors conclude that they identified a role for chromatin-independent functions of Ezh2. The term of chromatin-independent function seems an ambiguous. Since Ezh2, Suz12 and Eed form PRC2 to catalyze H3K27me3, it seems that an appropriate conclusion could be relevant to PRC2-independent function of Ezh2 in this study. To further refine the conclusion, authors may need to consider additional supportive evidence, such as:

1) Whether loss of Ezh2 in NKT cells affects the expression of Suz12 and Eed.

*We have now included the data showing that Suz12 is not affected by the loss of Ezh2 (Figure EV2c).*

2) Whether loss of Suz12 or Eed in NKT cells affects the expression of Ezh2.

*Our new western blot data indeed shows that Ezh2 is affected by the loss of Suz12 and Eed in thymocytes (Figure EV2b).*

*Both of these pieces of new data support a chromatin-independent function for Ezh2 in regulating NKT cell development.*

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Referee #3.

The referee was positive about our manuscript and they asked one major question:

The in vivo approach to test this hypothesis was not conclusive, though. The attempted experiment (retroviral transduction of wild type and mutant PLZF), however, would have interrogated PLZF protein levels rather than Ezh2-dependent modification of PLZF in these cells. A rather direct approach, analyzing PLZF in mutant cells or in cells derived from mutant mice would have been preferred. It is possible that cell numbers are of no help and that is a reason for the authors to have skipped such an approach, but this should have been discussed. Certainly, were the experiment to be technically feasible it should be attempted.

*It is unclear exactly what the reviewer is asking with this question. The experiment that they appear to be referring to was already performed in Figure 3 in which we showed that PLZF protein levels are high in ex vivo derived thymic Ezh2$^{cKO}$ NKT cells when compared to WT counterparts. We wanted to build on this to show whether alteration of the Ezh2 methylation site on PLZF led to higher protein levels and altered NKT cell development similar to that observed in Figure 3. Although the effects in Figure EV5 are subtle they indeed support this hypothesis and the potential caveats are mentioned in both the results and discussion sections.*

Despite this caveat, the findings are important in exposing regulatory functions of Polycomb products beyond the usual interest on chromatin modification(s).

Minor:

It is a bit surprising that reporting a non-histone methylation function for Ezh2 there is hardly any discussion about it or its relation to other protein methylation events.

*We have now added a paragraph in the discussion to discuss this in more detail.*

While for the authors it may be straightforward that PLZF is the product of the Zbtb16 locus, it would be better if this was indicated somewhere in the text describing results in Fig. 3.

 

*This has now been added to the text.*

References by Smyth GK 2004 or Xie et al. are incomplete.

*This has been corrected.*

It is the authors' manuscript but the closing paragraph in the discussion relates little to the core of the paper and does not add much in general.

*This paragraph has been replaced by a more relevant summary.*

2nd Editorial Decision 16 January 2017

Thank you for the submission of your revised manuscript to our editorial offices. I apologize for the delay in getting back to you, which is due to the Christmas holidays during which our editorial office was closed. We have now received the reports from the two referees that were asked to re-evaluate your study that you will find enclosed below. As you will see, both referees support the publication of your manuscript in EMBO reports. Referee #3 has a final comment. If you can add data to address his point, we ask you to add these in a final revised version. However, as also the referee indicates, we do not consider this critical for acceptance of the paper. Further, I have a few editorial requests.

Please format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

Please also remove the summary and the key findings from the manuscript main text. I have saved these and will provide them to the publisher upon acceptance/export.

Please add up to five key words (below the running title) and a conflict of interest statement (below the author contributions) to your manuscript.

Please send us a complete author checklist. There should be no blank fields. If the question is not relevant to your research, please write NA (non applicable).

In the manuscript you mention Supplementary Table 1 and Supplementary Table 2, which as I understand refers to Dataset 1 and Dataset 2. Please change the callouts to "Dataset 1" and "Dataset 2" and remove the legends for supplementary tables from the manuscript text.

Finally, the figure legend for Figure EV2 mentions two times (b). Please correct and/or combine this.

REFEREE REPORTS
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Referee #2: Authors have addressed all my questions. I have no further comments.

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Referee #3: The authors replied satisfactorily to the minor points.

Regarding the main query, it seems I didn't explain myself. I meant to obtain direct evidence of the proposed modification of PLZF/ZBTB16 in NK vs mutant cells. Indeed, an estimation of PLZF accumulation in mutant cells was provided in Fig. 3. On the other hand supplementary figure EV5 provide and indirect answer (mutant PLZF should be stabilized) but the magnitude of the increased levels is perhaps too subtle.

 

The thing is that all the evidence for a stabilization mechanism derives from a surrogate system, on 293 cells. I'm aware that the system is easier to work with and therefore used too often to provide biochemical support for one or another mechanism. It is just that, were to be cell-context dependent mechanisms involved there is no guarantee that these are faithfully represented in such a system. I don't know how feasible it would have been to detect altered mobility by western blot or even mass-spectrometry identification of methylated PLZF in wild type NK cells. Certainly, confidence in the suggested mechanism if not the strongest is reasonable, and at this time, for publication purposes, it does not appear a critical experiment.

2nd Revision - authors' response 18 January 2017

In addition to making the requested editorial changes, the authors submitted the following response:

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Referee #3 asked one final question:

Regarding the main query, it seems I didn't explain myself. I meant to obtain direct evidence of the proposed modification of PLZF/ZBTB16 in NK vs mutant cells. Indeed, an estimation of PLZF accumulation in mutant cells was provided in Fig. 3. On the other hand supplementary figure EV5 provide and indirect answer (mutant PLZF should be stabilized) but the magnitude of the increased levels is perhaps too subtle. The thing is that all the evidence for a stabilization mechanism derives from a surrogate system, on 293 cells. I'm aware that the system is easier to work with and therefore used too often to provide biochemical support for one or another mechanism. It is just that, were to be cell-context dependent mechanisms involved there is no guarantee that these are faithfully represented in such a system. I don't know how feasible it would have been to detect altered mobility by western blot or even mass-spectrometry identification of methylated PLZF in wild type NK cells. Certainly, confidence in the suggested mechanism if not the strongest is reasonable, and at this time, for publication purposes, it does not appear a critical experiment.

*We agree that this is an important point, however, due to the difficulty in obtaining enough material from primary NKT cells we have so far been unsuccessful in generating such data. In Figure EV4b we did perform IP of PLZF from wild type and Ezh2 deficient NKT cells and observed decreased methylation of PLZF. This supports the 293T data that showing that PLZF is indeed methylated by Ezh2.*

*We look forward to your response.*

3rd Editorial Decision 22 January 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

 

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

**A- Figures**

**1. Data**

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

**2. Captions**

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

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| http://www.ebi.ac.uk/ega | EGA |
| http://biomodels.net/ | Biomodels Database |
| http://biomodels.net/miriam/ | MIRIAM Guidelines |
| http://jjj.biochem.sun.ac.za | JWS Online |
| http://oba.od.nih.gov/biosecurity/biosecurity_documents.html | Biosecurity Documents from NIH |
| http://www.selectagents.gov/ | List of Select Agents |

**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? | We expected a greater than 50% difference between the mean of wild type and knock out mice with similar variance between samples. Therefore we calculated that we need a minimum of 4 mice/group to obtain a p value of <0.05 with 95% power. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | We expected a greater than 50% difference between the mean of wild type and knock out mice with similar variance between samples. Therefore we calculated that we need a minimum of 4 mice/group to obtain a p value of <0.05 with 95% power. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No samples were excluded |
| 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 was used |
| For animal studies, include a statement about randomization even if no randomization was used. | No randomization was used |
| 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 |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | No blinding was used |
| 5. For every figure, are statistical tests justified as appropriate? | Yes the statistical tests are appropriate. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Yes |
| Is there an estimate of variation within each group of data? | The variation between each group of data is similar. |
| 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). | All information about antibody clone names and companies they were purchased from is included in the materials and methods of the 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. | 293T cells were originally ordered from ATCC and are routinely tested for mycoplasma contamination. |

* 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. | Mus Musculus. C57BL/6, Ezh2fl/fl, Eedfl/fl, Suz12fl/fl, and Cd1d-/- mice. The floxed strains were crossed to Cd4cre mice. All mice lines have been maintained on a C57BL/6 (Ly5.2) background, maintained under SPF conditions and were used between 4 and 10 weeks of age. Both male and female mice were used. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | Animal experiments were in accordance with the guidelines of the Walter and Eliza Hall Institute Animal Ethics Committee. |
| 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. | We have consulted the ARRIVE guidelines and believe we have adequately reported the relevant aspects of our animal studies. |

**E- Human Subjects**

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |

| | |
|---|---|
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

**F- Data Accessibility**

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | GSE92548 |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | NA |
| 22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

**G- Dual use research of concern**

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|---|---|
| 23. 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 |