

RIG-I Antiviral Signaling Drives Interleukin-23 Production and Psoriasis-like Skin Disease

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Transaction Report:

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

Editor: Roberto Buccione

1st Editorial Decision

28 November 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the Reviewers whom we asked to evaluate your manuscript.

I again sincerely apologise for the very significant delay in reaching a decision on your manuscript. In this case, we first experienced significant difficulties in securing expert and willing Reviewers. I eventually only managed to secure two reviewers. Further to this, the evaluations were delivered with much regrettable delay.

I am proceeding based on the two evaluations obtained so far as further delay cannot be justified. However, I have also consulted with an external expert to inform my final decision.

As you will see, reviewer 1 is quite negative and while not very detailed, notes that the main conclusions are not supported by the data. Reviewer #2 is more positive, but also raises a similar basic concern and asks for further experimentation that might be unfeasible within a reasonable time frame (about 3 months) and, most importantly, with no guarantee of a successful outcome. After further discussion with my colleagues and the advisor, we agreed a revision would be need to be quite extensive and potentially requiring a significant length of time, with an inevitably uncertain outcome.

Given these fundamental concerns and the overall lack of enthusiasm by the Reviewers and our

advisor, I have no choice but to return the manuscript to you at this stage.

I wish to add that, considered the potential interest of these findings, we would have no objection to consider a new manuscript on the same topic if at some time in the near future you have obtained data that would considerably strengthen the message of the study and address the Reviewers' concerns. Please consider, however, that if you were to send a new manuscript this would be treated as a new submission.

At this stage of analysis, though, I am sorry to have to disappoint you. I nevertheless hope, that the reviewer comments will be helpful in your continued work in this area.

***** Reviewer's comments *****

Referee #1 (Remarks):

In this manuscript Zhu et al. claim that the psoriasiform phenotype induced by repeated IL-23 injection is dependent on viral dsRNA triggering of RIG-I with induction of endogenous IL-23. The manuscript has several problems. In particular, the link between IL-23 injection, RIG-I activation and the role of microbiota in the induction of psoriasiform inflammation via endogenous IL-23 is not convincingly demonstrated.

Major issues:

- 1) The fact that psoriasis induced by repetitive IL-23 injection requires endogenous IL-23 is not demonstrated. Does IL-23 injection into IL-23^{-/-} mice induce less acanthosis?
- 2) What is the mechanism that links IL-23 injection to RIG-I activation by viral 5'ppp-dsRNA? IL-23 upregulates RIG-I in SPF mice but not GF mice (Fig. 1G). How is this explained?
- 3) Is there a constitutive lack of RIG-I expression in GF mice or is it a lack of upregulation due to lack of activation in the absence of microbial dsRNA? What is the role of gut versus skin microbiota in this, in other words, can the reconstitution of skin microbiota restore RIG-I expression and development of psoriasiform skin lesions?
- 4) The conclusion that the microbiota is essential in the pathophysiology of the disease cannot be drawn by the data shown.

Minor issues:

- 5) The development of skin acanthosis upon il-23 injection is completely abrogated in GF mice but not in RIG-I^{-/-} mice. In fact, RIG-I^{-/-} mice seem to develop significant acanthosis. What is the reason for this discrepancy?
- 6) In supplementary Figure 2 the authors propose a model in which CD11c DC are activated by viral nucleic acids through both RIG-I and, surprisingly, TLR7/8 which has not been addressed in this study. Would this explain issue (5)?

Referee #2 (Comments on Novelty/Model System):

I think the findings are interesting - but the outcomes are limited in what they present - and they need to validate their findings in a second psoriasis-like actor model - such as Imiquimod, which is also IL-23 dependent. I have noted this in my comments to the authors.

Referee #2 (Remarks):

This is a very interesting paper that presents the argument that activation of RIG-I (which has recently been identified as a psoriasis susceptibility gene) by 5'ppp-dsRNA, directly causes the production of IL-23 and triggers psoriasis-like skin disease in mice. The authors present interesting data in germ free animals suggesting that commensals may contribute to the activation of IL-23 production via DCs via NFκB signaling. The text is extremely well written, and the story well presented. The figures, however, don't always align with the strong interpretation of the data, as the improvement in the skin phenotype appears frequently to be quite modest.

The work would be strengthened if the authors could address the following issues:

Do they see the same results using a second mouse model of acute-initiated psoriasiform skin

inflammation that is IL-23 dependent - i.e. can they demonstrate similar outcomes using imiquimod-elicited changes in their germ free conditions and in their KO model?

There has been much discussion in the skin community that intradermal IL-23 actually better models atopic dermatitis rather than psoriasis - the authors need to accommodate this discussion in their interpretation of their results.

What happens with WT bone marrow is transplanted into the KO mice, and then treated with either intradermal-IL-23 or imiquimod?

The outcomes the authors present currently are limited, additional outcomes would enhance their findings and interpretation. What happens to skin IL-17A? What happens to the the numbers and activation of DCs and T cells in the skin?

The method for analyzing acanthosis seems odd. The images presented show that there is still significant skin inflammation - i.e. the acanthosis of the skin is still present. Can they measure the intrafollicular epidermal thickness measures using average length (in microns) across the entire skin section (i.e. multiple measures between the basement membrane and the stratum corneum (not including any scale)? They can use Adobe photoshop to do this with the measure tool and calibrating it with a micrometer.

The authors need to discuss Dan Kaplan's recent work showing the nerve-DC-IL-23 interactions are critical for candidiasis - and also put those findings into context with theirs. It's possible that there is a neural influence on their outcomes as well, this has not been discussed.

How do the authors control for changes in ear thickness that simply happen as a result of the mouse ear anatomy and how close the ear skin is to the head? I.e. skin distal to the head is much thinner than ear skin closer to the head and can artificially inflate differences in ear thickness that are independent of the experimental manipulation.

1st Revision - authors' response

11 January 2017

Reviewer #1:

1. The fact that psoriasis induced by repetitive IL-23 injection requires endogenous IL-23 is not demonstrated. Does IL-23 injection into IL-23^{-/-} mice induce less acanthosis?

Re: It is a great question. To follow the reviewer's suggestion, we performed experiments by injecting recombinant IL-23 to ears of IL-23^{-/-} mice. We found that IL-23^{-/-} mice exhibited less severity of psoriasis-like skin inflammation compared with wild-type (WT) mice, including ear thickness and other disease features which is consistent with the study that targeting IL-23 results in clinical improvement in psoriasis (Kopp, Riedl et al., 2015) (Supplementary Fig. 1).

2. What is the mechanism that links IL-23 injection to RIG-I activation by viral 5'-ppp-dsRNA? IL-23 up-regulates RIG-I in SPF mice but not GF mice (Fig. 1G). How is this explained?

Re: We appreciate this constructive question raised by the reviewer. In our study we observed that besides the synthetic ligand of RIG-I 5'-ppp-dsRNA, IL-23 is also able to induce up-regulation of RIG-I, suggesting that IL-23 is possibly involved in host antiviral responses via regulating RIG-I expression (Fig. 3E). We further found that activation of RIG-I was able to induce endogenous IL-23p19 expression via NF-kappa B signaling (Fig. 7B and Fig. 7F). However, the detailed molecular mechanism that links IL-23 to RIG-I activation is subjected to another study.

SPF condition means there is only specific pathogen free but still exists some other microorganisms, suggesting that the presence of microorganism is essential for the activation of RIG-I. By contrast,

there is free of all microorganism in GF conditions, and in such conditions only IL-23 injection is not sufficient to sustain the endogenous IL-23 production without RIG-I activation by viruses. We have discussed it in the section of Discussion. **Page 16, line 6-11.**

3. Is there a constitutive lack of RIG-I expression in GF mice or is it a lack of up-regulation due to lack of activation in the absence of microbial dsRNA? What is the role of gut versus skin microbiota in this, in other words, can the reconstitution of skin microbiota restore RIG-I expression and development of psoriasiform skin lesions?

Re: It is a very nice question. The lack of up-regulation of RIG-I is likely due to lack of RIG-I activation triggered by microbial dsRNA because we observed there was very similar low levels of RIG-I expression in skin of GF and SPF mice when both treated by PBS (Fig. 1G).

It was published that the gut microbiota promotes psoriasis-like skin disease in imiquimod (IMQ)-induced murine model of psoriasis (Zakostelska, Malkova et al., 2016). To address whether skin microbiota (in our study particularly, RNA viruses) is critical for RIG-I expression and development of psoriasis-like skin lesions, we used IMQ to induce skin inflammation in RIG-I^{-/-} mice. We found that the disruption of RIG-I-mediated RNA virus infection attenuated psoriasis-like skin inflammation in RIG-I^{-/-} mice (Supplementary Fig. 3). Moreover, the IMQ treatment was able to up-regulate RIG-I expression in skin of WT mice (Figure 3F). Together, our data suggest that not only 5'-ppp-dsRNA, but also IL-23 and IMQ treatment are able to up-regulate RIG-I expression, and RIG-I-mediated antiviral signaling is critical for the development of psoriasis-like skin disease in IL-23- and IMQ-induced mouse models.

4. The conclusion that the microbiota is essential in the pathophysiology of the disease cannot be drawn by the data shown.

Re: The reviewer is right that we can not draw the conclusion that the microbiota is essential in the pathophysiology of psoriasis. However, in the current study we suggest that the RIG-I-mediated antiviral signaling triggers IL-23p19 expression and promotes psoriasis-like skin disease in both IL-23- and IMQ-induced mouse models of psoriasis (Fig. 4 and Supplementary Fig. 3). We have modified our writings in the revised manuscript accordingly. Page 13, line 12-13.

5. The development of skin acanthosis upon IL-23 injection is completely abrogated in GF mice but not in RIG-I^{-/-} mice. In fact, RIG-I^{-/-} mice seem to develop significant acanthosis. What is the reason for this discrepancy?

Re: We thank this very helpful question raised by the reviewer. We propose the fact that the RIG-I^{-/-} mice had some disease characteristics might be due to the RIG-I pathway is a critical, but not the only pathway in the pathogenesis of psoriasis as stimulation of the antiviral pattern-recognition receptors such as TLR7/TLR8 with IMQ, a synthetic agonist, is sufficient to trigger psoriasis-like skin inflammation in mice (van der Fits, Mourits et al., 2009).

6. In supplementary Figure 2 the authors propose a model in which CD11c DCs are activated by viral nucleic acids through both RIG-I and, surprisingly, TLR7/8 which has not been addressed in this study. Would this explain issue (5)?

Re: We have followed the reviewer's suggestion. We used IMQ to induce skin inflammation in RIG-I^{-/-} mice. We found that the disruption of RIG-I-mediated RNA virus infection slightly, but

significantly attenuated psoriasis-like skin inflammation in RIG-I^{-/-} mice (**Supplementary Fig. 3**).

Reviewer #2 (Remarks):

This is a very interesting paper that presents the argument that activation of RIG-I (which has recently been identified as a psoriasis susceptibility gene) by 5'ppp-dsRNA, directly causes the production of IL-23 and triggers psoriasis-like skin disease in mice. The authors present interesting data in germ free animals suggesting that commensals may contribute to the activation of IL-23 production via DCs via NFκB signaling. The text is extremely well written, and the story well presented. The figures, however, don't always align with the strong interpretation of the data, as the improvement in the skin phenotype appears frequently to be quite modest.

The work would be strengthened if the authors could address the following issues:

1. Do they see the same results using a second mouse model of acute-initiated psoriasiform skin inflammation that is IL-23 dependent - i.e. can they demonstrate similar outcomes using imiquimod-elicited changes in their germ free conditions and in their KO model?

Re: This is a very helpful suggestion. It was just published that the psoriasis-like skin inflammation induced by IMQ is attenuated in GF conditions (Zakostelska et al., 2016). We have followed the reviewer's comments by treating IMQ in RIG-I^{-/-} mice in SPF conditions. We found that the RIG-I^{-/-} mice showed amelioration of psoriasis-like skin inflammation when treated with IMQ (**Supplementary Fig. 3**). These mice had thinner ear thickness, less ki67 positive staining and decreased expression levels of IL-23p19 and IL-17 (**Supplementary Fig. 3**). Additionally, we found that repeated injections of ears with IL-23 induced a profound increase of IL-17 protein levels in the SPF mice, whereas IL-23 injection did not induced IL-17 expression in the GF mice (**Fig. 11**).

2. There has been much discussion in the skin community that intradermal IL-23 actually better models atopic dermatitis rather than psoriasis - the authors need to accommodate this discussion in their interpretation of their results.

Re: We have followed the reviewer's helpful suggestion and discussed it in the section of Discussion. **Page 14, line 3-6**.

3. What happens with WT bone marrow is transplanted into the KO mice, and then treated with either intradermal-IL-23 or imiquimod?

Re: In fact, we have showed these data in **Supplementary Fig. 4**. When RIG-I^{-/-} mice were reconstituted with WT bone marrow, these mice exhibited comparable levels of severity of psoriasis-like skin inflammation compared with WT mice.

4. The outcomes the authors present currently are limited, additional outcomes would enhance their findings and interpretation. What happens to skin IL-17A? What happens to the numbers and activation of DCs and T cells in the skin?

Re: We followed the reviewer's comments, and investigated the IL-17 protein levels in skin by ELISA, and confirmed that after repeated injections of IL-23, the WT mice had a dramatic increase in IL-17A expression when compared with the PBS treated group (**Supplementary Fig. 2D**). Meanwhile, the RIG-I^{-/-} mice only slightly elevated compared with WT mice (**Supplementary Fig.**

2D). By performing staining we found there was a pronounced increase in the expression of CD3 and CD11c in WT mice compared to RIG-I^{-/-} mice (Supplementary Fig. 2A-C).

5. The method for analyzing acanthosis seems odd. The images presented show that there is still significant skin inflammation - i.e. the acanthosis of the skin is still present. Can they measure the intrafollicular epidermal thickness measures using average length (in microns) across the entire skin section (i.e. multiple measures between the basement membrane and the stratum corneum (not including any scale)? They can use Adobe photoshop to do this with the measure tool and calibrating it with a micrometer.

Re: We followed the reviewer's comments, and corrected it by measuring the thickness between the basement membrane and the stratum corneum by using average length in microns.

6. The authors need to discuss Dan Kaplan's recent work showing the nerve-DC-IL-23 interactions are critical for candidiasis - and also put those findings into context with theirs. It's possible that there is a neural influence on their outcomes as well, this has not been discussed.

Re: It is a very nice suggestion, and we have discussed it in the discussion. Page 14, line 16-19.

7. How do the authors control for changes in ear thickness that simply happen as a result of the mouse ear anatomy and how close the ear skin is to the head? I.e. skin distal to the head is much thinner than ear skin closer to the head and can artificially inflate differences in ear thickness that are independent of the experimental manipulation.

Re: It is correct that skin distal to the head is much thinner than ear skin closer to the head. To make our data as precise as possible, we choose to measure the same site in the middle of the mouse ears and use the relative value to evaluate ear thickness.

References

Kopp T, Riedl E, Bangert C, Bowman EP, Greisenegger E, Horowitz A, Kittler H, Blumenschein WM, McClanahan TK, Marbury T, Zachariae C, Xu D, Hou XS, Mehta A, Zandvliet AS, Montgomery D, van Aarle F, Khalilieh S (2015) Clinical improvement in psoriasis with specific targeting of interleukin-23. *Nature* 521: 222-6

van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, Cornelissen F, Mus AM, Florencia E, Prens EP, Lubberts E (2009) Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 182: 5836-45

Zakostelska Z, Malkova J, Klimesova K, Rossmann P, Hornova M, Novosadova I, Stehlikova Z, Kostovcik M, Hudcovic T, Stepankova R, Juzlova K, Hercogova J, Tlaskalova-Hogenova H, Kverka M (2016) Intestinal Microbiota Promotes Psoriasis-Like Skin Inflammation by Enhancing Th17 Response. *PLoS One* 11: e0159539

2nd Editorial Decision

24 February 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I again apologise for the very unusual delay in providing you with a decision.

Unfortunately we could not retrieve an opinion from reviewer 1 notwithstanding repeated emails and phone calls. While we find this inexcusable, we eventually went back to reviewer 2 to ask his/her opinion on your rebuttal to reviewer 1's comments.

Based on the enclosed report from reviewer 2 and his/her statement that your actions on reviewer 1's concerns are satisfactory, I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please take appropriate action on reviewer 2's final requests. I agree that to do so would improve the overall quality of your discussion and presentation

2) Every published paper includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short description as well as 2-5 one-sentence bullet points that summarise the key NEW findings of the paper. The bullet points should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information. Please use the passive voice. Please attach this information in a separate file or send them by email, we will incorporate it accordingly. We also encourage the provision of striking image or visual abstract to illustrate your article. If you do, please provide a jpeg file 550 px-wide x 400-px high.

3) Please upload your figures as individual files

4) We encourage the publication of source data, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or at least the key gels used in the manuscript and/or source data sets for relevant graphs? The files should be labeled with the appropriate figure/panel number, and in the case of gels, should have molecular weight markers; further annotation may be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

5) We now mandate that all corresponding authors list an ORCID digital identifier. You may acquire one through our web platform upon submission and the procedure takes <90 seconds to complete. We also encourage co-authors to supply an ORCID identifier, which will be linked to their name for unambiguous name identification.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

Finally, I hope that the inevitable frustration caused by this delay is somewhat tempered by the positive outcome.

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System):

Thanks for validating your findings in a second model.

Referee #2 (Remarks):

The authors have done what I requested.

Issues still needing a bit of attention are detailed below:

They need to still better discuss the nuances of IL-23 as an AD model vs a psoriasis model and incorporate the recent JACI paper - by Emma Guttman - Ewald et al , 2016

They need to emphasize that their effects hold up across TWO ACUTE models of elicited skin inflammation - IL-23 and imiquimod - and make the wording less strong on Imiquimod being psoriasis - its not - it's a murine model of some aspects of psoriasis-like disease

They need to add a comment that they are unclear what will happen in a chronic mouse model of

psoriasis and its possible the results could differ - reflecting criticality at initiation and perhaps less during reversal and remission

If they are going to quote the Riol Blanco paper - they really should include the first paper that showed this phenomena - the Ostrowski et al JID paper. - which demonstrates disease improvement rather than prevention...and again the authors are encouraged to also include The Dan Kaplan work looking at nerves and DC interactions.

2nd Revision - authors' response

28 February 2017

Reviewer #2 (Comments on Novelty/Model System):

Thanks for validating your findings in a second model.

Referee #2 (Remarks):

The authors have done what I requested.

Issues still needing a bit of attention are detailed below:

1. They need to still better discuss the nuances of IL-23 as an AD model vs a psoriasis model and incorporate the recent JACI paper - by Emma Guttman - Ewald et al, 2016.

Re: We followed the reviewer's comments, and discussed the nuances of IL-23 as an AD model vs a psoriasis model in the section of DISCUSSION (Page 14, line 2-8).

2. They need to emphasize that their effects hold up across TWO ACUTE models of elicited skin inflammation - IL-23 and imiquimod - and make the wording less strong on Imiquimod being psoriasis - its not - it's a murine model of some aspects of psoriasis-like disease.

Re: We are so grateful for the reviewer's suggestion, and we corrected our wording by using "IMQ-induced psoriasis-like mouse model" in the whole manuscript. We also emphasized that the mouse model we used in our research only represented the acute phase of psoriasis (Page 16, line 5-6).

3. They need to add a comment that they are unclear what will happen in a chronic mouse model of psoriasis and its possible the results could differ - reflecting criticality at initiation and perhaps less during reversal and remission.

Re: It is a very nice suggestion, and we have added a comment to clarify that the phenomena in chronic models need to be further investigated (Page 16, line 5-6) and RIG-I is probably a critical trigger rather than a regulator in remission in psoriasis (Page 16, line 14).

4. If they are going to quote the Riol Blanco paper - they really should include the first paper that showed this phenomena - the Ostrowski et al JID paper. - which demonstrates disease improvement rather than prevention...and again the authors are encouraged to also include The Dan Kaplan work looking at nerves and DC interactions.

Re: We have followed the reviewer's helpful suggestions and discussed it in the section of DISCUSSION (Page 14, line 18-19).

3rd Editorial Decision

01 March 2017

Unfortunately, there are a few final items for you to act upon, which we noticed only on your last submission:

1) Thank you for providing source data for some of the figures. I note however, that that there does not appear to be a perfect match between Fig 3 panel E and the corresponding blot in the source data file. Please correct/explain. Also, please provide source data as one file per each figure.

2) As per our Author Guidelines, the description of all reported data that includes statistical testing

must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05'). You may wish to collect all P values in a separate table and refer to it in the legends. I am sorry that I omitted to mention this before, but do note that this requirement is clearly mentioned in the author guidelines (<http://embomolmed.embopress.org/authorguide#datapresentationformat>)

3) We suggest that you remove all siRNA and primer sequences from the main text and collect them in a separate table, which you can refer to in the text.

4) Please provide TIFF rather than JPEG files

5) Please try to make the scale bars a bit more clearly visible

6) Please make the supplementary figures Expanded View figures instead (<http://embomolmed.embopress.org/authorguide#expandedview>) and carefully update figure callouts in the manuscript

7) References cannot feature more than 20 authors et al. Please correct.

8) We are still missing the Author Checklist (<http://embomolmed.embopress.org/authorguide>).

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible. The sooner you send as the revised files, the sooner I can accept!

3rd Revision - authors' response

02 March 2017

Thank you so much for your efforts to accept our revised manuscript (**EMM-2016-07027**) for publication in *EMBO Molecular Medicine*. We think that we have satisfactorily amended the manuscript, and loaded all required files to the Journal.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Honglin Wang

Journal Submitted to: EMBO Molecular Medicine

Manuscript Number: EMM-2016-07027

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 χ^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.

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).

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?	Page 35, Page 36, Page 37, Page 38, Page 39, Page 40, Page 41, Page 42, Page 43
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Page 35, Page 36, Page 37, Page 38, Page 39, Page 41, Page 42, Page 43
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Figure 1H and 1I, due to the degradation of some samples
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.	Page 18
For animal studies, include a statement about randomization even if no randomization was used.	Page 18
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.	Page 21
4.b. For animal studies, include a statement about blinding even if no blinding was done	Page 21
5. For every figure, are statistical tests justified as appropriate?	Page 25
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Page 25
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	Page 25

C- Reagents**USEFUL LINKS FOR COMPLETING THIS FORM**<http://www.antibodypedia.com><http://1degreebio.org><http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo><http://grants.nih.gov/grants/olaw/olaw.htm><http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm><http://ClinicalTrials.gov><http://www.consort-statement.org><http://www.consort-statement.org/checklists/view/32-consort/66-title><http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tur><http://datadryad.org><http://figshare.com><http://www.ncbi.nlm.nih.gov/gap><http://www.ebi.ac.uk/ega><http://biomodels.net/><http://biomodels.net/miriam/><http://ijb.biochem.sun.ac.za>http://oba.od.nih.gov/biosecurity/biosecurity_documents.html<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Page 20-22
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 24

* 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.	Page 18
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 18
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.	Page 18

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Page 19
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.	Page 19
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'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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. Examples: Primary Data Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CRA/5 of TR. Protein Data Bank 4O26 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 Biomedelis (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

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.	NA
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