EMBO reports

Osteoclasts adapt to physioxia perturbation through DNA demethylation

Keizo Nishikawa, Shigeto Seno, Toshitada Yoshihara, Ayako Narazaki, Yuki Sugiura, Reito Shimizu, Junichi Kikuta, Reiko Sakaguchi, Norio Suzuki, Norihiko Takeda, Hiroaki Semba, Masamichi Yamamoto, Daisuke Okuzaki, Daisuke MOTOOKA, Yasuhiro Kobayashi, Makoto Suematsu, Haruhiko Koseki, Hideo Matsuda, Masayuki Yamamoto, Seiji Tobita, Yasuo Mori, and Masaru Ishii
DOI: 10.15252/embr.202153035

Corresponding author(s): Keizo Nishikawa (kenishik@mail.doshisha.ac.jp) , Yasuo Mori (mori@sbchem.kyoto-u.ac.jp), Masaru Ishii (mishii@icb.med.osaka-u.ac.jp)

Review Timeline:

Submission Date:	9th Apr 21
Editorial Decision:	19th May 21
Revision Received:	29th Jul 21
Editorial Decision:	2nd Sep 21
Revision Received:	3rd Sep 21
Accepted:	16th Sep 21

Editor: Martina Rembold

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 19th May 2021

Dear Dr. Nishikawa

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, all referees also point out a number of concerns and suggestions on how to substantiate the current data set. The oxygen probe used should be better validated and supported by alternative methods to measure oxygen. Two referees point out that EPO might have direct effects on osteoclasts and osteoblasts and either such a contribution should be ruled out or the data removed. The involvement of metabolites should be tested.

From the referee comments it is clear that a major revision will be required before the study can be considered for publication. Yet, given the overall positive evaluation and constructive comments, we would like to give you the chance to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the 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.

We invite you to submit your manuscript within three months of a request for revision. This would be August 19th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

***IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.***

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please list the accession numbers and database for the RNA-seq, MBD-seq, and GeneChip data in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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

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])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal 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 .

10) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify whether these are technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

In this study, Nishikawa and colleagues investigate the role of oxygen availability on osteoclastogenesis. Using in vivo hypoxia sensors, transgenic mouse models, and in vitro assays, the authors propose a model whereby low oxygen levels hamper osteoclast formation independently of HIF activity and energy metabolism. Instead, they claim that hypoxia decreases the DNA demethylation activity of TET enzymes, which in turn reduces NFATc1 signaling necessary for osteoclastogenesis.
This study provides novel insight on the role of environmental oxygen for osteoclast function, although some of the claims are insufficiently supported by the presented data. The main concerns are detailed below.

Major concerns:
1- Several studies, which were correctly cited in the manuscript, have investigated the effect of hypoxia on osteoclast formation and found that osteoclastogenesis was increased when cultured in low oxygen. While the authors rightfully argue that the used culture conditions might differ and likely not fully mimic the in vivo situation, Arnett et al. (J Cell Physiol, 2003) assessed the effect of a range of oxygen concentrations, including 5% and 2% pO2. In sharp contrast to this study, they found that osteoclastogenesis was increased. Can the authors explain this apparent discrepancy?
2- To assess the effect of chronic hypoxia on bone and osteoclast function in vivo, a transgenic Epo-/-;Tg[3.3K-Epo3'] mouse model was used. Yet, no evidence was provided that hypoxia was induced in osteoclasts using this model. In addition, Epo is known to exert direct effects on both osteoclasts (Hiram-Bab S, FASEB J 2015) and osteoblasts (Suresh S, FASEB J 2020), making it difficult to correctly interpret the effect on bone tissue. The added value of this model is therefore questioned, unless the mentioned issues are addressed
3- While the effect on osteoclast number after hypoxic culture or TET inactivation is evident, it remains unclear which cellular properties were affected. Is there an effect on survival, proliferation, fusion and/or activity, and is this similar in the different models?
4- As a mechanism, the authors propose that hypoxia reduces TET activity, although TET-mediated effects on osteoclastogenesis were analyzed using genetic TET inactivation. The authors should mimic some of the functional effects induced by hypoxia using TET activity blockers (e.g. 2-HG, succinate, ...) to fully prove that it is TET activity.
5- NFATc1 overexpression fully rescues the decrease in osteoclast formation upon TET deletion, although this strategy was not sufficient to completely restore osteoclastogenesis during hypoxia, arguing that decreasing oxygen levels also exerts other inhibitory effects. The authors should therefore discuss this issue that other oxygen responding systems are likely involved.

Minor comments:
1- In the in vivo-low oxygen model, what is the effect on osteoblast-related parameters?
1- For all in vivo models, representative pictures should be provided to support the histological analysis of osteoclast and osteoblast number/activity.
2- For the loss and gain-of-function models, please show validation of the changes in the protein of interest, both at the mRNA and protein level.
3- Figure 3D,E: please also provide the data in WT animals, and test the differences in bone mass as such
4- What is the effect of TET/NFATc1 overexpression in control conditions?
5- Figure S10: please provide a representative TRAP staining picture of the control condition

6- Scale bars are missing in Figure 1B,D, 4A,B and S1A
7- A recent study by Thienpont and colleagues also demonstrates that, in cancer cells, hypoxia reduces TET activity independently of HIF (Thienpont B, Nature 2016), which might be worth referring to.

Referee #2:

The manuscript from Nishikawa K. et al aimed to quantify cellular oxygen in osteoclast in vivo using two-photon phosphorescence. They described an altered osteoclastogenesis dependent of the TET enzymes, which play a role in oxygen dependent DNA methylation. The concept and results of the manuscript are interesting. The notion of HIF independency makes the findings quite different than what was expected. I would however complete the study with the following points to strength the manuscript before acceptance to EMBO reports.

1- The level of oxygen is a critical point where their findings is based since TETs appear activated at a lower KM range compare to PHD-HIFs axis. The authors should mention in vivo only for the figure 1D, where all the rest is in vitro. Moreover, the correlation between 98-55 spO2 to 5-2% O2 in vitro should be better explained.
2- The authors forgot to include that HIFs can also play a role in osteoclastogenesis in an independent manner. The control of this pathway should be included with culture at normal oxygen incubation for the wildtype and the knock-out used.
3- The EPO deficiency model is not helpful for the manuscript. As it is raising more questions than answers. For the manuscript clarity, I would suggest removing it.
4-in figure 3, the discrepancy between in vivo and in vitro results is not supporting that HIFs have no role in their findings. In vitro and in vivo wildtype littermate as controlled should be shown.
5- in figure 5 a better characterization of TET 1,2 ,3 expression at mRNA and protein levels during osteoclastogenesis and at different oxygen tension conditions should be performed.
6- Analysis of osteoclastogenesis of TET deficient cell has not been done in different oxygen conditions. It would have been interesting to see if the phenotype is oxygen dependent.
7- Instead of overexpression of NFATc1, which in fact will compensate any other mechanism defect in osteoclast, defining the importance of DNA methylation would highly upgrade the molecular mechanism of Tet1/2 in osteoclast.

Referee #3:

This manuscript presents several findings in relation to osteoclast and bone formation and their relationship with oxygen tension. The authors present a relatively novel probe that can estimate intracellular oxygen using imaging, they measure in vivo oxygen levels in the mouse, investigate the effects on hypoxia on osteoclast differentiation in vitro and in vivo, then rule out HIFs but demonstrate a role for TET2 and TET3 in the oxygen effect presented. This involves a variety of mouse models and crosses. As such the work presented here is very extense. Overall, it is very interesting but there is a lot to digest.

Some points:
The oxygen probe use needs more documentation, such as a wider range of oxygen tensions and also the dynamycs of the probe. Ideally, another mechanisms to demonstrate oxygen changes

should be presented although the reviewer appreciates that no other quantitative method exists apart from invasive methods, so an indirect methods would be acceptable

In the in vitro models, the reviewer does not undertand why these primary cells were cultured initially at 18%, surely all primary cells should be cultured at 5% to avoid oxidated damage. This might explain some of the discrepancies between the old studies and this one.

Also every cells in an body will have its normoxic level, cells that have been cultured at atmospheric oxygen have since aclimitised but primary cells do not have this time period.

In the mice experiments, why the use of 14% for 10 days. Most hypoxic experiments in mice are done at 10%.

Although energy metabolism does not seem involved, metabolites might still be involved, such as succinate or fumarate. Can the authors rule these out? At least this should be discussed.

Finally, since the genetics indicate TET2/3 are involved, the use of a broad spectrum dioxygenase inhibitor should be used to demonstrate that this is simply not a loss of the protein.

Minor point, in the abstract the authors state that the cellular response to hypoxia has not been explored. This is not really true, since intensive knowledge exists on this topic. Maybe the authors want to rephrase this statement.

Another interesting statement is: "Although routine cell culture in room air is one of the mainstays of life science research, in vitro analysis considering a physioxia condition may have made our experiments more robust and increased the likelihood that
these findings have in vivo relevance". In this reviewer experience, this only applies to primary cells. In addition, the findings leading to the vast of our knowledge on the oxygen pathway were conducted in such conditions and they have been shown to be highly relevant to physiology and medicine, so some care in needed while making such statement. Especially based on a single approach to measure normoxic levels of cells in vivo

Responses to the reviewers' comments on EMBOR-2021-53035-T

We are grateful to all the reviewers for their insightful comments and valuable suggestions. To address all the issues raised by the reviewers, we have incorporated additional data after performing further experiments and have carefully revised the manuscript. We believe that the revised manuscript has taken into consideration, essentially, all suggestion made by the reviewers. Please find our responses to each comment in a point-by-point manner below.

Referee #1:

Comment 1: Several studies, which were correctly cited in the manuscript, have investigated the effect of hypoxia on osteoclast formation and found that osteoclastogenesis was increased when cultured in low oxygen. While the authors rightfully argue that the used culture conditions might differ and likely not fully mimic the in vivo situation, Arnett et al. (J Cell Physiol, 2003) assessed the effect of a range of oxygen concentrations, including 5% and 2% pO2. In sharp contrast to this study, they found that osteoclastogenesis was increased. Can the authors explain this apparent discrepancy?

We agree with the reviewer that it is important to explain this discrepancy. This discrepancy may be attributed to the different culture conditions for *in vitro* osteoclast differentiation between two studies. We performed *in vitro* osteoclast differentiation based on the monoculture method using RANKL and M-CSF, and examined the effect of hypoxia on osteoclast differentiation by culturing bone marrow cells under the condition of 5% and 2% oxygen. On the other hand, in the study of Arnett *et al.*, bone marrow cells were cultured in the presence of high concentration of dexamethasone, prostaglandin E2, and active form of vitamin D in addition to RANKL and M-CSF. Since factors such as dexamethasone, prostaglandin E2, and vitamin D may be responsible for this discrepancy, we examined the effect of hypoxia in the presence of these factors. However, even in the presence of these factors, we observed that osteoclast formation was significantly impaired at 2% oxygen compared to that in 5% (Appendix Fig S1B in the revised manuscript). Furthermore, the formation of osteoclasts was inhibited under 1% oxygen compared to that under 5% oxygen. These results are in accordance with the results from a previous study(Fukuoka *et al*, 2005).

In the study by Arnett *et al.*, changing the oxygen concentration from 5% to 2% promoted osteoclast formation, while 0.2% and 1% oxygen concentration inhibited osteoclast formation. Moreover, they cultured bone marrow cells on ivory discs, whereas we cultured the cells on conventional tissue culture dishes. The ivory discs could be responsible for causing the shift in oxygen concentration threshold to attenuate osteoclast formation from 2% to 0.2% or 1%. Unfortunately, since ivory discs are not commercially available due to prohibition of international trade of ivory under the Washington convention, it is currently impossible to conduct the experiments using ivory discs. At first glance, one might think that culturing osteoclasts on ivory discs would be closer to physiological conditions than culturing on conventional tissue culture dishes, but bone is not similar to ivory in terms of its composition, structure, and the presence of osteocytes. Ivory is mainly made up of dentin and mineral, while bone is composed of various materials, such as collagen and mineral. Furthermore, bone, unlike ivory, contains osteocytes that are involved in the regulation of osteoclasts. Therefore, the increase in osteoclastogenesis when the oxygen concentration was changed from 5% to 2% is thought to be a phenomenon unique to the cells cultured on ivory discs, which do not provide accurate physiological conditions. We have added the data in Appendix Fig S1B and described these points in the figure legend of Appendix Fig S1B in the revised manuscript.

Comment 2: To assess the effect of chronic hypoxia on bone and osteoclast function in vivo, a transgenic Epo-/-;Tg[3.3K-Epo3'] mouse model was used. Yet, no evidence was provided that hypoxia was induced in osteoclasts using this model. In addition, Epo is known to exert direct effects on both osteoclasts (Hiram-Bab S, FASEB J 2015) and osteoblasts (Suresh S, FASEB J 2020), making it difficult to correctly interpret the effect on bone tissue. The added value of this model is therefore questioned, unless the mentioned issues are addressed.

Following the reviewer's and editor's comments, we have removed the results for Epo-/-;Tg[3.3K-Epo3'] mouse from the revised manuscript.

Comment 3: While the effect on osteoclast number after hypoxic culture or TET inactivation is evident, it remains unclear which cellular properties were affected.

Is there an effect on survival, proliferation, fusion and/or activity, and is this similar in the different models?

Following the reviewer's comments, we have added a dataset including the effects of hypoxia or TET inactivation on proliferation, survival, and terminal differentiation. We examined the effects on cell proliferation and apoptosis by performing flow cytometry analysis. However, no obvious difference in the number of BrdU-positive and Annexin V-positive bone marrow-derived monocyte/macrophage precursor cells (BMMs) was observed by changing oxygen concentration from 5% to 2% (Appendix Fig S1C and D in the revised manuscript). Furthermore, the number of both types of cells was comparable between the control and $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs (Fig EV3D and E in the revised manuscript). These results suggest that both cell proliferation and apoptosis of osteoclast precursors were not affected by hypoxia or TET inactivation. We have described these points in the revised manuscript (page 17, line 332-335).

Comment 4: As a mechanism, the authors propose that hypoxia reduces TET activity, although TET-mediated effects on osteoclastogenesis were analyzed using genetic TET inactivation. The authors should mimic some of the functional effects induced by hypoxia using TET activity blockers (e.g. 2-HG, succinate, ...) to fully prove that it is TET activity.

Following the reviewer's suggestion, we used a cell-permeable 2-hydroxyglutarate derivative, octyl-2HG, as a TET-specific inhibitor, and IOX1, as a broad-spectrum inhibitor of 2-oxoglutarate-dependent oxygenase, to study the effect of loss of TET activity. No obvious difference in the number of BrdU-positive and Annexin V-positive BMMs was observed among control, and octyl-2HG-treated and IOX1-treated BMMs (Appendix Fig S8C and D in the revised manuscript). On the other hand, treatment with octyl-2HG and IOX1 decreased the number of TRAP-positive multinucleated cells (Appendix Fig S8A in the revised manuscript). Furthermore, the expression of osteoclastogenic genes such as *Prdm1*, *Nfatc1*, *Ctsk* and *Acp5* was significantly decreased in the octyl-2HG-treated and IOX1-treated BMMs (Appendix Fig S8B in the revised manuscript). Therefore, it is likely that the effects of octyl-2HG and IOX1 are likely to mimic the effects of hypoxia. This information is included in the revised manuscript (page 17, line 339-page 18, line 346).

Comment 5: NFATc1 overexpression fully rescues the decrease in osteoclast formation upon TET deletion, although this strategy was not sufficient to completely restore osteoclastogenesis during hypoxia, arguing that decreasing oxygen levels also exerts other inhibitory effects. The authors should therefore discuss this issue that other oxygen responding systems are likely involved.

As the reviewer correctly pointed out, the overexpression of NFATc1 significantly restored osteoclastogenesis under hypoxia, but the extent of the recovery was low. The effects of hypoxia did not fully mimic the effects of TET inactivation. Since various oxygen-dependent enzymes were expressed in both osteoclast precursors and mature osteoclasts (Appendix Fig S2B in the revised manuscript), hypoxia may inhibit osteoclastogenesis by reducing the activity of these enzymes, which are a part of a downstream pathway independent of NFATc1 regulation. We have described these points in the revised manuscript (page 20, line 389-391).

Comment 6: In the in vivo-low oxygen model, what is the effect on osteoblast-related parameters?

Following the reviewer's suggestion, we have added data on osteoblast-related parameter (Fig 2C in the revised manuscript). The hypoxia signaling pathway is involved in the promotion of osteoblastogenesis(Wang *et al*, 2007), but there were no obvious differences in the number of osteoblasts between the control and hypoxic mice in our experiment, wherein the mice were exposed to 14% oxygen for 10 days. The effect of hypoxia on osteoblasts may be manifested in the mice exposed to hypoxia for longer periods. This information is included in the revised manuscript (page 11, line 207-211).

Comment 7: For all in vivo models, representative pictures should be provided to support the histological analysis of osteoclast and osteoblast number/activity.

Following the reviewer's comments, we have provided representative images to support bone histomorphometric analysis. For the histological analysis of *Tet* knockout mice, we have added images of TRAP staining and toluidine blue staining used to measure the number of osteoclasts and osteoblasts, respectively (Fig 5C and EV4B in the revised manuscript), and images of calcein double labelling used to measure the

activity of osteoblasts (Fig EV4C in the revised manuscript). In contrast, for the histological analysis of *Hif* knockout mice and the mice exposed to hypoxic air, we have added images of toluidine blue staining used to measure the number of both osteoclasts and osteoblasts (Fig 2B in the revised manuscript). In this experiment, only static bone morphometric parameters were measured because we could not inject calcein into the mice, which were being kept in a locked hypoxic chamber, as it is technically difficult to measure dynamic parameters such as bone formation using calcein double labelling.

Comment 8: For the loss and gain-of-function models, please show validation of the changes in the protein of interest, both at the mRNA and protein level.

Following the reviewer's suggestion, we have shown the expression of both mRNA and protein to validate the change in the protein of interest. In $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs, both mRNA and protein of Tet2, Tet3, Blimp1, NFATc1, Ctsk, and TRAP were reduced (Fig EV3C in the revised manuscript). In $Hif1a_{Rank}^{-/-}$; $Hif2a_{Rank}^{-/-}$ BMMs, both mRNA and protein of HIF-1α, HIF-2α, Blimp1, NFATc1, Ctsk, and TRAP were also reduced (Fig 3A and C in the revised manuscript). In BMMs overexpressing NFATc1 and TET variants, exogenous proteins were overexpressed (Appendix Fig 7B, 9C, D and E in the revised manuscript).

Comment 9: Figure 3D,E: please also provide the data in WT animals, and test the differences in bone mass as such

We apologize that the layout of the Figures was confusing. We have already provided the data from control littermates in Fig 2C of the original manuscript. To avoid confusion, we have revised the figure to include data from both control and $Hif1a_{Rank}^{-/-}$; $Hif2a_{Rank}^{-/-}$ mice (Fig 2 in the revised manuscript).

Comment 10: What is the effect of TET/NFATc1 overexpression in control conditions?

Forced expression of NFATc1 led to an increased number of TRAP-positive cells, whereas there was no difference in osteoclast formation between mock control and Tet2-overexpressed cells. This data has been added to Fig 6F and H, and Appendix Fig S7B and S9D in the revised manuscript.

Comment 11: Figure S10: please provide a representative TRAP staining picture of the control condition

Following the reviewer's comments, we have provided representative images for the control condition (Appendix Fig S7B in the revised manuscript).

Comment 12: Scale bars are missing in Figure 1B,D, 4A,B and S1A

Thank you for pointing this out. We have added scale bars in the all images and have listed the images with scale bars below (Fig 1B, D and F; 2A and B; 3B, 4A and B; 5A-C and E; 6F and H; Fig EV1A; EV4A-C; EV5C; Appendix Fig S1A and B, S6, S7B, S8A, and S9E in the revised manuscript).

Comment 13: A recent study by Thienpont and colleagues also demonstrates that, in cancer cells, hypoxia reduces TET activity independently of HIF (Thienpont B, Nature 2016), which might be worth referring to.

Thank you for pointing out this important reference. We have followed the reviewer's advice and cited the reference.

Referee #2:

Comment 1: The level of oxygen is a critical point where their findings is based since TETs appear activated at a lower KM range compare to PHD-HIFs axis. The authors should mention in vivo only for the figure 1D, where all the rest is in vitro. Moreover, the correlation between 98-55 spO2 to 5-2% O2 in vitro should be better explained.

We are very sorry for the lack of description for the correlation mentioned in the comments. The arterial oxygen saturation, $sp\mathrm{O}_2$, of the mice under normal air, measured by a pulse oximeter, ranged from 98% to 95%. In the mice, the *in vivo* oxygen tension of osteoclasts was determined to be 36.4 ± 0.7 mmHg (ca. 5%) using two-photon phosphorescence lifetime imaging microscopy. In contrast, when the mice were exposed to low oxygen air by changing oxygen concentration from 21% to 10%, the $sp\mathrm{O}_2$ of the mice gradually shifted from 98% to 55%, depending on several conditions, such as oxygen concentration and rate of flow, and the rate of breathing and heart beat of the mice. In these mice, the *in vivo* oxygen tension of osteoclasts was 27.8 ± 0.8, 24.4

± 0.6, 21.8 ± 0.8, and 17.4 ± 0.6 (ca. 2%), at spO_2 85%, 75%, 65%, and 55%, respectively. This information has been included in the revised manuscript (page 10, line 181-183).

Comment 2: The authors forgot to include that HIFs can also play a role in osteoclastogenesis in an independent manner. The control of this pathway should be included with culture at normal oxygen incubation for the wildtype and the knock-out used.

We are very sorry that the layout of the Figures was confusing. We have already provided the data for wild-type control in Fig 1E of the original manuscript. To avoid confusion, we have changed the figure to include data from both control and $Hif1a_{Rank}^{-/-}$; $Hif2a_{Rank}^{-/-}$ mice (Fig 2 in the revised manuscript). Since the protein expression of both HIF-1α and HIF-2α was negligible in osteoclast precursors and mature osteoclasts under atmospheric air in the humidified incubator, we examined the effect of both hypoxia and HIF knockout on osteoclastogenesis by culturing bone marrow-derived monocyte/macrophage precursor cells (BMMs) under 5% and 2% oxygen. While no obvious differences in the formation of TRAP-positive multinucleated cells as well as the expression of osteoclastogenic genes between control and $Hif1a_{Rank}^{-/-}$; $Hif2a_{Rank}^{-/-}$ BMMs were observed, the expression of canonical HIF-target genes, such as *Slc2a1* and *Pkm2* decreased in $Hif1a_{Rank}^{-/-}$; $Hif2a_{Rank}^{-/-}$ BMMs (Fig 3A–C in the revised manuscript).

Comment 3: The EPO deficiency model is not helpful for the manuscript. As it is raising more questions than answers. For the manuscript clarity, I would suggest removing it.

Following the reviewer's and editor's comments, we have omitted the results of Epo-/-;Tg[3.3K-Epo3'] mouse from the revised manuscript.

Comment 4: in figure 3, the discrepancy between in vivo and in vitro results is not supporting that HIFs have no role in their findings. In vitro and in vivo wildtype littermate as controlled should be shown.

We are very sorry that Fig 2 and 3 in the original manuscript were confusing. We have already provided the data for wild-type littermate in Fig 2C and D of the original

manuscript. To avoid confusion, we have changed the figure to include data from both control littermates and *Hif1a*$_{Rank}^{-/-}$; *Hif2a*$_{Rank}^{-/-}$ mice (Fig 2 and 3 in the revised manuscript). Both control littermates and *Hif1a*$_{Rank}^{-/-}$; *Hif2a*$_{Rank}^{-/-}$ mice exposed to hypoxic air exhibited a similar phenotype, i.e., high bone mass accompanied with decreased number of osteoclasts. Consistent with the *in vivo* data, hypoxia decreased in *in vitro*-generated osteoclasts from control and *Hif1a*$_{Rank}^{-/-}$; *Hif2a*$_{Rank}^{-/-}$ BMMs. Thus, these *in vivo* and *in vitro* results suggest that hypoxia inhibits osteoclastogenesis in a HIFs-independent manner.

Comment 5: in figure 5 a better characterization of TET 1,2 ,3 expression at mRNA and protein levels during osteoclastogenesis and at different oxygen tension conditions should be performed.

Following the reviewer's suggestion, we have added the data of both mRNA and protein expression of Tet1, Tet2 and Tet3 in osteoclast precursors and mature osteoclasts at different oxygen concentrations (Fig EV3A-C, and EV5A and B in the revised manuscript).

Comment 6: Analysis of osteoclastogenesis of TET deficient cell has not been done in different oxygen conditions. It would have been interesting to see if the phenotype is oxygen dependent.

Following the reviewer's comment, we performed *in vitro* osteoclast differentiation assay for *Tet2*$_{Rank}^{-/-}$; *Tet3a*$_{Rank}^{+/-}$ BMMs under different oxygen concentrations (Fig EV5C in the revised manuscript). Consistent with the results observed under atmospheric air in the humidified incubator, the formation of TRAP-positive multinucleated cells from *Tet2*$_{Rank}^{-/-}$; *Tet3a*$_{Rank}^{+/-}$ BMMs was also significantly decreased under 5% oxygen. However, there was no difference in the number of TRAP-positive multinucleated cells under 2% oxygen, as a few TRAP-positive multinucleated cells were formed from both control and *Tet2*$_{Rank}^{-/-}$; *Tet3a*$_{Rank}^{+/-}$ BMMs. This information has been included in the revised manuscript (page 17, line 331-332).

Comment 7: Instead of overexpression of NFATc1, which in fact will compensate any other mechanism defect in osteoclast, defining the importance of DNA

methylation would highly upgrade the molecular mechanism of Tet1/2 in osteoclast.

In this study, the analysis of RNA-seq and MeDIP-seq data from wild-type and $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs, revealed 25 genes as putative TET target genes. Of these genes, we performed gain of function analysis of the following genes: *Prdm1*, *Prckh* and *Gsap*, in $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs, but their forced expression could not rescue the differentiation of $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs to osteoclasts (data not shown). Thus, the functional TET-target genes in osteoclasts remains unclear. The reason for this may be the complexity of epigenetic regulation by TETs. TETs carry out conversion of 5-methylcytosine to several other modified forms of cytosine, such as 5-hydroxymethylcytosine, 5-formycytosine, and 5-carboxycytosine. Recent studies have proposed that these cytosine derivatives act as epigenetic marks whose function is different from cytosine and 5-methylcytosine. Therefore, MeDIP-seq analysis may not be a comprehensive approach to identify putative TET-target genes. In addition, recently, evidences has emerged that supports the role of TET-mediated oxidation of 5-methylcytosine in mRNA and tRNA(Shen *et al*, 2021; Shen *et al*, 2018). Thus, to further elucidate the molecular mechanism underlying TET-mediated osteoclast regulation in detail, integrative multi-omics analysis may be necessary for the identification of potential TET target genes. We agree with the reviewer's opinion, but this was out of the scope of this manuscript.

Referee #3:

Comment 1: The oxygen probe use needs more documentation, such as a wider range of oxygen tensions and also the dynamycs of the probe. Ideally, another mechanisms to demonstrate oxygen changes should be presented although the reviewer appreciates that no other quantitative method exists apart from invasive methods, so an indirect methods would be acceptable.

We are very sorry for limited explanation of the property and dynamic range of the oxygen probe. We have previously demonstrated that the phosphorescence probe, BTPDM1, distributes to most of tissues in the body within 2 hours after injection and accumulates in the cells of these tissues for at least 24 hours (PMID: 25634116). Furthermore, phosphorescence imaging using BTPDM1 determined that oxygen level in hypoxic tumors, renal cortex, and hepatic lobules in live animals ranged from 0.8 to 60

mmHg (PMID: 25634116, PMID: 26644023, PMID: 33273499), showing that BTPDM1 has high sensitivity with a wide dynamic range. Furthermore, oxygen level of renal cortex was ca. 50 mmHg, which was almost equivalent to the level measured by oxygen electrodes. Thus, our phosphorescence imaging technique using BTPDM1 is a reliable method to measure intracellular oxygen tension in tissues *in vivo*. This information has been included in the revised manuscript (page 25, line 493-498).

Several methods to assess oxygen perturbation in different tissues are available. Immunohistochemistry for pimonidazole adducts and HIF-α accumulation is widely used for analyzing hypoxia within tissue, but it is neither quantitative nor indicative of oxygen tension. Electrochemical electrodes are used to measure oxygen tension in real-time, for e.g., the tip of micro-electrode is positioned directly in the target tissue, and the electrode in the gas analyzer measures oxygen using the collected blood. However, these invasive procedures are not suitable for the measurement of oxygen tension in hard tissue. On the other hand, non-invasive methods to quantitatively assess oxygen tension *in vivo* have been developed based on magnetic resonance techniques including magnetic resonance imaging (MRI), nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR)(Roussakis *et al*, 2015). These methods have both advantages and limitations in terms of applicable targets, spatial resolution, tissue permeability, convenience, and reversibility, but they are not suitable for the measurement of oxygen tension at cellular level. Taken together, we agree with the reviewer's opinion that oxygen tension in osteoclasts should be confirmed using alternative methods, but except phosphorescence imaging using BTPDM1, there are no other methods to measure oxygen tension in hard tissue at single cell level. We have described these points in the revised manuscript (page 7, line 128- page 8, line 144).

Comment 2: In the in vitro models, the reviewer does not undertand why these primary cells were cultured initially at 18%, surely all primary cells should be cultured at 5% to avoid oxidated damage. This might explain some of the discrepancies between the old studies and this one. Also every cells in an body will have its normoxic level, cells that have been cultured at atmospheric oxygen have since aclimitised but primary cells do not have this time period.

We are very sorry that the description was confusing. By 18% we meant that the culture was under atmospheric air in a humidified incubator. Generally, while atmospheric air contains ca. 21% oxygen concentration, oxygen concentration is calculated to be 18.6% in an incubator after the addition of both water vapor and carbon dioxide (PMID: 29032224). Indeed, we confirmed that oxygen concentration in our humidified incubator was 18% by using a portable fiber optic oxygen meter. To avoid confusion, we have changed the description as 'atmospheric air in the humidified incubator' or 'air' instead of 18%. This information is included in the revised manuscript (page 10, line 193 and Appendix Fig S1A).

Furthermore, following the reviewer's comment, we performed *in vitro* osteoclast differentiation assay from $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs under 5% and 2% oxygen in addition to atmospheric air in a humidified incubator as shown in the original manuscript. Consistent with the results observed under atmospheric air in the humidified incubator, the formation of TRAP-positive multinucleated cells from $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs was also significantly decreased under the condition of 5% oxygen (Fig EV5C in the revised manuscript).

Comment 3: In the mice experiments, why the use of 14% for 10 days. Most hypoxic experiments in mice are done at 10%.

We agree with the reviewer's opinion that most experiments in mice under hypoxic conditions are done at 10% oxygen concentration. Initially, we attempted to examine the *in vivo* effect of hypoxia on mice under 10% or less oxygen concentration, but the mice were exposed for 10 days, and did not consume food and displayed hypoactivity and lean mass with less bone mass. Therefore, alternatively, we exposed the mice to 14% oxygen concentration. La Paz, the capital of Bolivia, is located at an altitude of 3,700 meters, which is considered to be the highest altitude at which humans can live on a daily basis, and the oxygen concentration at 3,700 meters is 14%. This information has been included in the revised manuscript (page 24, line 471-475).

Comment 4: Although energy metabolism does not seem involved, metabolites might still be involved, such as succinate or fumarate. Can the authors rule these out? At least this should be discussed.

As the reviewer correctly pointed out, although we showed that hypoxia did not

affect ATP level and NAD(P)H metabolism in osteoclasts *in vivo*, we could not exclude the possibility that hypoxia could have affected the level of other metabolites. To assess this possibility, we assayed metabolites in osteoclast precursors under 5% and 2% oxygen concentration using IC-MS. As a result, while hypoxia did not affect the levels of glycolysis metabolites, the levels of TCA cycle metabolites were significantly changed (Fig EV2 in the revised manuscript). In particular, 2-oxoglutarate level in BMMs under 2% oxygen concentration was significantly increased, suggesting that hypoxia may impair the activity of the 2-oxoglutarate-consuming enzyme. This information has been included in the revised manuscript (page 15, line 285-291).

Comment 5: Finally, since the genetics indicate TET2/3 are involved, the use of a broad spectrum dioxygenase inhibitor should be used to demonstrate that this is simply not a loss of the protein.

Following the reviewer's suggestion, to study the effect of loss of TET activity, we used IOX1, a broad-spectrum inhibitor of 2-oxoglutarate-dependent oxygenase, in addition to octyl-2-hydroxyglutarate (Octyl-2HG), as a TET-specific inhibitor, which was pointed out by reviewer #1. There was no difference in the number of BrdU-positive and Annexin V-positive BMMs among control, IOX1-treated, and octyl-2HG-treated BMMs (Appendix Fig 8C and D in the revised manuscript). On the other hand, treatment with IOX1 and octyl-2HG decreased the number of TRAP-positive multinucleated cells (Appendix Fig 8A in the revised manuscript). Furthermore, the expression of osteoclastogenic genes such as *Prdm1*, *Nfatc1*, *Ctsk*, and *Acp5* was significantly decreased in octyl-2HG-treated and IOX1-treated BMMs (Appendix Fig 8B in the revised manuscript). These effects of IOX1 and octyl-2HG are likely to mimic the phenotype of $Tet2_{Rank}^{-/-}$; $Tet3a_{Rank}^{+/-}$ BMMs, suggesting that TET activity is important for osteoclast regulation. This information has been included in the revised manuscript (page 17, line 339 and page 18, line 346).

Comment 6: Minor point, in the abstract the authors state that the cellular response to hypoxia has not been explored. This is not really true, since intensive knowledge exists on this topic. Maybe the authors want to rephrase this statement.

Thank you for pointing out this mistake in our description. We have changed the description as follows: However, since quantitation of the partial pressure of cellular

oxygen *in vivo* is challenging, the extent of oxygen perturbation *in situ* and its cellular response remains underexplored.

Comment 7: Another interesting statement is: "Although routine cell culture in room air is one of the mainstays of life science research, in vitro analysis considering a physioxia condition may have made our experiments more robust and increased the likelihood that these findings have in vivo relevance". In this reviewer experience, this only applies to primary cells. In addition, the findings leading to the vast of our knowledge on the oxygen pathway were conducted in such conditions and they have been shown to be highly relevant to physiology and medicine, so some care in needed while making such statement. Especially based on a single approach to measure normoxic levels of cells in vivo.

We agree with the reviewer that the sentence is strong, which we did not intend. The sentence now reads as follows (page 22, line 447- page 23, line 450): Although routine cell culture in room air is one of the mainstays of life science research, primay cell-based *in vitro* analysis under physioxia may have made our experiments more robust and increased the likelihood that these findings have *in vivo* relevance.

References

Fukuoka H, Aoyama M, Miyazawa K, Asai K, Goto S (2005) Hypoxic stress enhances osteoclast differentiation via increasing IGF2 production by non-osteoclastic cells. *Biochem Biophys Res Commun* 328: 885-894

Roussakis E, Li Z, Nichols AJ, Evans CL (2015) Oxygen-Sensing Methods in Biomedicine from the Macroscale to the Microscale. *Angew Chem Int Ed Engl* 54: 8340-8362

Shen H, Ontiveros RJ, Owens MC, Liu MY, Ghanty U, Kohli RM, Liu KF (2021) TET-mediated 5-methylcytosine oxidation in tRNA promotes translation. *J Biol Chem* 296: 100087

Shen Q, Zhang Q, Shi Y, Shi Q, Jiang Y, Gu Y, Li Z, Li X, Zhao K, Wang C *et al* (2018) Tet2 promotes pathogen infection-induced myelopoiesis through mRNA oxidation. *Nature* 554: 123-127

Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, Bouxsein ML, Faugere MC, Guldberg RE, Gerstenfeld LC *et al* (2007) The hypoxia-inducible factor alpha pathway

couples angiogenesis to osteogenesis during skeletal development. *J Clin Invest* 117: 1616-1626

1st Revision - Editorial Decision 2nd Sep 2021

Dear Dr. Nishikawa

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and request only minor changes to the text.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Please change the heading "Competing Interests" to "Conflict of Interest".

- Author contributions: Please specify the contribution of Yuki Sugiura and Prof. Makoto Suematsu in this paragraph.

- We recommend adding a bit more vertical space between Western blots in the individual figure panels to more clearly separate the individual blots.

- Please add callouts to the panels of Fig. 2, Fig. EV2, EV3 and EV5 where appropriate. Also callouts to Fig. 3B, C are missing.

- Appendix: Please define the number of technical or biological repeats in the legends of Fig. S1A-D, Fig. S2, Fig. S4, Fig. S8, and Fig. S9 A, B, D

- Appendix Fig S8: For A and B the definition of **P is missing, while it is not required for panel C, D. Alternatively, you could summarize this information for all panels as "Data information: Scale bar, 100 um. Data denote mean… "

- Appendix table S1 is rather small in size and should be enlarged.

- In the legend of Appendix Fig. S2 you mention data deposition to the Genome Network platform. If you deposited a dataset there, this should be added to the Data availability paragraph.

- Appendix Figure S1B: please format the reference to Fukuoka, 2005 #125 and to Arnett, 2003 #124.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.
- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership and I have introduced some suggestions. Please amend the text further to clearly highlight your findings also to non-experts.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors responded to the questions and comments by performing additional experiments and adapting the text. I do not have extra comments.

Referee #2:

The manuscript has been highly updated by the authors after the revision. It is now really strong and comprehensive. The manuscript is of high interest for the readership of EMBO reports.

I would nevertheless have a a minor comment that might easily be address:
the discrepancy of the in vitro hypoxic results with the previous manuscript from Arnett et al has been nicely explained by the authors in the rebuttal letter but might be also discussed in the manuscript in more details.

I have no further comments.

Referee #3:

IN my opininion, the authors have provided a much improved version and addressed the vast majority of my concerns

Responses to the reviewers' comments on EMBOR-2021-53035-V2

We thank the editor for the kind consideration of our manuscript and the reviewer for their comment which have greatly improved our manuscript. We believe that the revised manuscript has taken into consideration, essentially, all comment made by the editor and the reviewer. Please find our responses to each comment in a point-by-point manner below.

I would nevertheless have a a minor comment that might easily be address:
the discrepancy of the in vitro hypoxic results with the previous manuscript from Arnett et al has been nicely explained by the authors in the rebuttal letter but might be also discussed in the manuscript in more details.

Following the reviewer's comment, we have changed the description in the figure legend of Appendix Fig S1B in the revised manuscript as follows: These results were similar to those from a previous study(Fukuoka *et al.*, 2005), but were contrary to those from a study wherein the cells were cultured on ivory discs, which showed that changing the oxygen concentration from 5% to 2% promoted osteoclast formation(Arnett *et al.*, 2003). However, since the study showed that changing the oxygen concentration from 2% to 0.2% inhibited osteoclast formation, the ivory discs could be responsible for causing the shift in oxygen concentration threshold to attenuate osteoclast formation.

Please change the heading "Competing Interests" to "Conflict of Interest".
be also discussed in the manuscript in more details.

Following the editor's instruction, we have changed the description from "Competing Interests" to "Conflict of Interest".

Author contributions: Please specify the contribution of Yuki Sugiura and Prof. Makoto Suematsu in this paragraph.

Following the editor's instruction, we have added the description for YS and MS.

We recommend adding a bit more vertical space between Western blots in the individual figure panels to more clearly separate the individual blots.

Following the editor's instruction, we have showed each individual blot clearly.

Please add callouts to the panels of Fig. 2, Fig. EV2, EV3 and EV5 where appropriate. Also callouts to Fig. 3B, C are missing.

Following the editor's instruction, we have added callouts to the each panel of all Figures. Due to figure callout order, the figure EV4 and EV5 have been replaced.

- Appendix: Please define the number of technical or biological repeats in the legends of Fig. S1A-D, Fig. S2, Fig. S4, Fig. S8, and Fig. S9 A, B, D

Following the editor's instruction, we have defined the number of biological repeats in the legends of all figures.

- Appendix Fig S8: For A and B the definition of **P is missing, while it is not required for panel C, D. Alternatively, you could summarize this information for all panels as "Data information: Scale bar, 100 um. Data denote mean... "

Following the editor's instruction, we have added the information. We also noticed that the legends of Figure 8AB and Figure 8CD were reversed, so we corrected them.

- Appendix table S1 is rather small in size and should be enlarged.

Following the editor's instruction, we have changed the Appendix table S1.

- In the legend of Appendix Fig. S2 you mention data deposition to the Genome Network platform. If you deposited a dataset there, this should be added to the Data availability paragraph.

Following the editor's instruction, we have added the description in the Data availability paragraph.

- Appendix Figure S1B: please format the reference to Fukuoka, 2005 #125 and to Arnett, 2003 #124.

Following the editor's instruction, we have formatted these references.

2nd Revision - Editorial Decision 16th Sep 2021

Dr. Keizo Nishikawa
Doshisha University
1-3 Tatara Miyakodani
Kyoto 610-0394
Japan

Dear Dr. Nishikawa,

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Keizo Nishikawa, Yasuo Mori and Masaru Ishii
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53035-T

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 iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	page 33
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	page 33
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	page 33
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 23
For animal studies, include a statement about randomization even if no randomization was used.	Page 23
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.	Yes, Page 23
4.b. For animal studies, include a statement about blinding even if no blinding was done	Page 23
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. I assessed it using GraphPad Prism.
Is there an estimate of variation within each group of data?	Yes.

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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).	Page 32
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 did not use cell lines in this study.

* 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 23
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 23
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 confirmed.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Page 34
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).	All data setsare registered in the Gene Expression Omnibus database.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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