THE EMBO JOURNAL

A Zeb1/MtCK1 Metabolic Axis Controls Osteoclast Activation and Skeletal Remodeling

Lingxin Zhu, Yi Tang, Xiao-Yan Li, Samuel A. Kerk, Costas A. Lyssiotis, Wenqing Feng, Xiaoyue Sun, Geoffrey E. Hespe, Zijun Wang, Marc P. Stemmler, Simone Brabletz, Thomas Brabletz, Evan T. Keller, Jun Ma, Jung-Sun Cho, Jingwen Yang, and Stephen J Weiss

DOI: 10.15252/embj.2022111148

Corresponding author(s): Stephen Weiss (sjweiss@umich.edu) , Lingxin Zhu (lingxin.zhu@whu.edu.cn)

Review Timeline:

Submission Date:	13th Mar 22
Editorial Decision:	27th Apr 22
Revision Received:	1st Sep 22
Editorial Decision:	20th Oct 22
Appeal Received:	24th Oct 22
Editorial Decision:	12th Jan 23
Revision Received:	29th Jan 23
Accepted:	2nd Feb 23

Editor: William Teale

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.)

Dear Prof. Weiss,

Thank you again for the submission of your manuscript entitled "A Zeb1/MtCK1 Metabolic Axis Controls Osteoclast Activation and Skeletal Remodeling" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, while referee 1 raises valid concerns that your data do not go far enough to excluding contributions from regulatory factors distinct from MTCK1, overall the feedback was positive.

Therefore, based on the interest that is expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. This will probably require additional lab work, as outlined in the reports of referees 3 and 1. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. I believe the concerns of the referees are reasonable and addressable and recommend we organize a brief Zoom chat to discuss the comments and go through any potential problems there may be in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Best regards,

William Teale

William Teale, Ph.D.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

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).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response 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 (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). 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.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed

under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

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

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

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

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can 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 or a single pdf per main figure 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 at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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 labelled 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.

11) 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 .

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Further instructions for preparing your revised manuscript:

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

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

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

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

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

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

Referee #1:

In the current study, Zhu and colleagues describe a regulatory axis that involves the TF ZEB1 and the mitochondrial creatine kinase 1 (MTCK1). More concretely the authors indicate that in osteoclasts ZEB1 is upregulated and regulates i.e. suppresses mitochondrial bioenergetics. Upon genetic deletion of ZEB1 in mice osteoclasts are hyperactive thus leading to decreased bone density.

The study is interesting but too descriptive.

In my opinion, the interpretation of the experimental findings and the resulting conclusions might be oversimplified. Moreover, the mechanistic details require deeper understanding.

Specific comments:

- In panels A and B of Fig. 1, the bioinformatic evaluation of BMDM-Osteoclast transcripts is shown and indicates that other processes and TF's are involved in the osteoclast differentiation. For example, oxidoreductase activity is more affected that DNA-binding. Given the monocyte-origin of these cells, it is likely that redox signaling i.e. NADPH oxidases are also very important for osteoclast function. This particularly important because ZEB1 and ZEB2 have been shown to be involved in redox regulation. Furthermore, other TFs known to affect mitochondrial biogenesis such as NFAT, are also regulated. It is therefore surprising that ZEB1 plays such a decisive, all-or-nothing role in osteoclast biology via regulation of only one enzyme i.e. MTCK1. The authors need to address these and other alternatives so that the manuscript is suitable for a journal such as EMBO Journal. At this stage the manuscript would be a better fit for a specialized journal.

- WM and fluorescent images throughout the text need to be quantified and statistically evaluated.

Referee #2:

Based on an unbiased transcriptomic approach, which identified that expression of the transcriptional repressor Zeb1 increases during the course of osteoclastogenesis, the authors generated a mouse model with myeloid-specific Zeb1 inactivation to uncover a previously unknown role of Zeb1 as a negative regulator of bone resorption. Through a comprehensive molecular analysis of Zeb1-deficient osteoclasts they identified increased expression of MtCK1 as a major driver of this phenotype and thereby uncovered a critical role of the creatine kinas axis for osteoclast-mediated bone resorption. All presented data are highly convincing and supported by different methodological approaches, including siRNA-knockdown, promoter binding studies, normalization by treatment with cyclocreatine, and confirmation of the data by experiments with human osteoclasts. The presentation of these novel and relevant data is truly excellent, and there is no need for further improvement. There is only one issue that the authors should consider, i.e. to add a schematic presentation summarizing the Zeb1-dependent mechanism that was uncovered.

Referee #3:

In their manuscript, Zhu and colleagues identify the transcriptional repressor Zeb1 as a key factor in regulating the bone-resorbing activity of osteoclasts (OCs) and show that it functions by down-regulating mitochondrial creatine kinase (MtCK) and the levels of its product, phosphocreatine.
Specifically, The authors identify Zeb1 as a gene that is up-regulated in mature OCs relative to bone marrow-derived macrophages (BMDMs), and that myeloid-specific knockout of Zeb1 in mice reduces bone mass due to increased bone resorption by OCs. The knockout OCs exhibit more prominent actin rings (sealing zones), which are central to resorptive activity, in correlation with increased activity of RhoA in the cells. Of note, differentiation of OCs is unaffected in these mice, as is bone matrix production by osteoblasts. The authors then identify MtCK, but not other forms of creatine kinase, as a target of the transcriptional repressor activity of Zeb1and establish that MtCK is up-regulated in the Zeb1-knockout OCs, along with increased mitochondrial respiration and ATP production in these cells. These changes are specific since mitochondrial structure, mitochondrial mass, DNA copy number, and levels of key mitochondrial complexes are unchanged. Importantly, these effects, as well as enhanced actin rings, are replicated in OCs that express exogenous MtCK, and also in wild-type OCs exposed to exogenous phosphocreatine. In line with these results, knockdown of MtCK in Zeb1-deficient OCs rescues the mitochondrial abnormalities. Importantly, the findings were replicated also in human monocyte-derived OCs in which expression of Zeb1 was partially down-regulated, while the expected opposite results were obtained upon partial down-regulation of MtCK.

This is a well-conceived study that was performed with care, and contains results that support the conclusions made. The study is presented in a clear manner and adequately discusses relevant literature. Important roles for the cytosolic brain form of creatine kinase in OCs were described previously by Chang et al, 2008 (quoted in the current manuscript), but the authors show that their findings are independent of that form.

In all, this manuscript describes a novel role for Zeb1 in regulating OC activity and skeletal structure through regulation of energy metabolism in OCs. OCs are known to require significant amounts of energy for their activity; this study uncovers a novel Zeb1-MtCK-phosphocreatine axis that helps control this key cellular activity, making it relevant to the broader cell biology community.

Major points:
1. While the Zeb1-MtCK-phosphocreatine axis is well-described, it is less clear how elevated levels of phosphocreatine, which are the product of the axis in the current study, promote the bone-resorbing activity of OCs. Is this driven by enhanced RhoA activity and actin ring formation? Are the RhoA and mitochondrial phenotypes directly connected in this respect? The data also suggest that the energy status of OCs can drive, as opposed to simply enable, their bone resorption activity. How does this fit in with other known mechanisms that regulate OC activity?
2. Page 5, 1st paragraph: the authors describe Zeb1 as present in cells that express proteins characteristic of mature OCs. Similarly, Figure 1 compares mature OCs with BMDMs. The authors should examine Zeb1 expression at several time points during the osteoclastogenic process in vitro. When does Zeb1 expression begin? Is MtCK expression correlated with Zeb1 expression throughout the process?
3. The authors show that the actin rings of Zeb1-deficient OCs occupy a larger fraction of the cell area (Fig. 3H). Additional data is required for better understanding of this. Is the size (or nuclear number) of Zeb1-deficient OCs changed? Is the number of actin rings per OC altered? Is the alphaVbeta3 integrin altered (localization, phosphorylation) in the mutant OCs?

Minor points:
1. The genetic background(s) of the mice used should be specified. Also, the status of the Zeb1 and Cre alleles of the of the littermate "wild-type" control mice (Figure 2) should be defined.
2. The nature of the catalytically-inactive MtCK C316G mutant should be noted (also) in the main text.
3. Legends to Figure 1 and Supp. Figure 1: There is some discrepancy between the panel indicators in the legend texts and in the figures.
4. Figure 2B and Supp. Figure 2B: To which bone type does the data refer?

5. Figure 6C: MtCK Western blot: assuming that the lower band represents the exogenous protein, why does it migrate differently from the endogenous one?
6. Enhanced expression of MtCK in human monocyte-derived OCs should be demonstrated also by protein blotting and not only by comparing immunofluorescence images (Supp. Fig. 4).
7. Supp. Figure 5: mature OCs are large fragile cells that might not do well in FACS analysis. Panel A shows images of mature OCs, while the Methods section (p. 19) describes use of OC precursors in this study. Can the authors clarify?
8. P.10, middle paragraph, speaks of "RhoA levels" - this may be misleading since it refers to RhoA activity or levels of active RhoA.

Responses to Reviewers

EMBOJ-2022-111148: "A Zeb1/Mitochondrial Creatine Kinase Metabolic Axis Controls Osteoclast Activation and Skeletal Remodeling" by Lingxin Zhu, Yi Tang, Xiao-Yan Li, Samuel A. Kerk, Wenqing Feng, Xiaoyue Sun, Geoffrey E. Hespe, Zijun Wang, Jingwen Yang, Jun Ma, Jung-Sun Cho, Marc P. Stemmler, Simone Brabletz, Thomas Brabletz, Evan T. Keller, Costas A. Lyssiotis, and Stephen J. Weiss

Reviewer #1:

General comments:

In the current study, Zhu and colleagues describe a regulatory axis that involves the TF ZEB1 and the mitochondrial creatine kinase 1 (MTCK1). More concretely the authors indicate that in osteoclasts ZEB1 is upregulated and regulates i.e. suppresses mitochondrial bioenergetics. Upon genetic deletion of ZEB1 in mice osteoclasts are hyperactive thus leading to decreased bone density. The study is interesting but too descriptive. In my opinion, the interpretation of the experimental findings and the resulting conclusions might be oversimplified. Moreover, the mechanistic details require deeper understanding.

We appreciate the constructive comments and believe our manuscript has been improved significantly after addressing each of the Reviewer's concerns and supplement more mechanistic details to help understand. Point-by-point responses are included below.

Specific comments:

1. In panels A and B of Fig. 1, the bioinformatic evaluation of BMDM-Osteoclast transcripts is shown and indicates that other processes and TF's are involved in the osteoclast differentiation. For example, oxidoreductase activity is more affected that DNA-binding. Given the monocyte-origin of these cells, it is likely that redox signaling i.e. NADPH oxidases are also very important for osteoclast function. This particularly important because ZEB1 and ZEB2 have been shown to be involved in redox regulation.

Indeed, our gene ontology analysis identified an enrichment in both DNA-binding transcription factor (TF) and oxidoreductase activities during normal osteoclastogenic programming (Fig 1A). However, transcription profiling of *Zeb1*-deleted osteoclasts doesn't reveal evident change of the essential TFs involved in osteoclastogenic programming, including *jdp2, pbx1, mycl, spic, fosl2,* and *prdm1* (Fig 1B; Fig 4A). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, primarily Nox2 and Nox4, as well as reactive oxygen species (ROS) can positively regulate osteoclast differentiation (*Blood* 106:852, 2005; *J Clin Invest* 123:4731, 2013; *Sci Rep* 6:38014, 2016; *Free Radic Biol Med* 132:67, 2019). In addition, Zeb1 has been reported to affect ROS production in cancer cells by transcriptionally upregulating *Slc16a4* and downregulating *Gpx4* gene expression (*Breast Cancer Res Treat* 188:329, 2021; *J Cell Sci* 412:113044, 2022). As such, we have assessed ROS and H_2O_2 production, as well as the gene expression of the essential NADPH oxidase family members, *Cybb* (encoding Nox2 protein) and *Nox4*, as well as *Slc16a4* (encoded Mct4 protein), and *Gpx4* (encoded Gpx4 protein) expression in $Zeb1^{\Delta M/\Delta M}$ osteoclasts. As shown in our revised text, ROS and H_2O_2 levels are comparable between wild-type and $Zeb1^{\Delta M/\Delta M}$ osteoclasts (Appendix Fig S4G and H), which is consistent with our observation that myeloid-specific depletion of *Zeb1* does not affect the osteoclast differentiation (i.e., the primary target of osteoclast-derived ROS described in the current literature

cited above). Likewise, gene expression levels of *Cybb/Nox2 and Nox4,* as well as *Slc16a4* and *Gpx4*, are indistinguishable between wild-type and *Zeb1*-deficient cells (Appendix Fig S4I). These data have been inserted in the revised text. We have also expanded the discussion and references regarding the role of Zeb1 in redox regulation to help clarify mechanistic details.

2. Furthermore, other TFs known to affect mitochondrial biogenesis such as NFAT, are also regulated. It is therefore surprising that ZEB1 plays such a decisive, all-or-nothing role in osteoclast biology via regulation of only one enzyme i.e. MTCK1. The authors need to address these and other alternatives so that the manuscript is suitable for a journal such as EMBO Journal. At this stage the manuscript would be a better fit for a specialized journal.

As described, Fig 2B lists 20 of the most highly expressed transcription factors during wild-type macrophage-osteoclast transition, including many of those previously linked to osteoclast differentiation, including *Nfatc1* (*Dev Cell* 3:889, 2002). These results, save for the inclusion of *Zeb1*, were not unexpected as these changes coincided with normal osteoclastogenesis. Of course, high expression of *Zeb1* does not intimate function, but we were surprised that *Zeb1* targeting yielded such a dramatic bone resorption phenotype *in vivo*.

To begin identifying potential Zeb1 targets capable of regulating bone resorbing activity, we conducted an unbiased transcriptome analysis comparing the expression profiles of wild-type *versus* $Zeb1^{\Delta M/\Delta M}$ osteoclasts. Given that Zeb1 is classically characterized as a transcriptional repressor (Stemmler et al., 2019; Vandewalle et al., 2009), transcriptome analysis indicated that among the top 10 upregulated genes in knockout cells, the mitochondrial creatine kinase gene, *Ckmt1* – an enzyme critical to mitochondrial oxidative activity and energy metabolism (Schlattner et al., 2006; Wallimann et al., 2011; Wallimann et al., 1992), was the most highly expressed transcript in $Zeb1^{\Delta M/\Delta M}$ osteoclasts (Fig 4A and B). We then validated that $Zeb1^{\Delta M/\Delta M}$ osteoclasts display markedly increased MtCK1 gene and protein levels while expression of the cytosolic creatine kinase, Ckb, remained unchanged (Fig 4E-H; Appendix Fig S4A). Though mitochondrial mass, mtDNA copy number, and OXPHOS protein expression are indistinguishable between wild-type and $Zeb1^{\Delta M/\Delta M}$ osteoclasts (Fig 5D; Appendix Fig S5A-D), the mitochondrial creatine kinase activity, PCr/Cr ratios, and mitochondrial respiration were remarkably increased in $Zeb1^{\Delta M/\Delta M}$ osteoclasts (Fig 5 A-C and E). More importantly, our conclusion that MtCK1 plays a dominant role in in $Zeb1^{\Delta M/\Delta M}$ osteoclasts was based on set of 4 independent results; the hyperactive bone resorptive phenotype in $Zeb1^{\Delta M/\Delta M}$ cells was normalized following either i) sh*Ckmt1* transduction that reduced MtCK1 levels to that found in wild-type osteoclasts or ii) the addition of the creatine kinase inhibitor, cyclocreatine (Fig EV 4E-K and 5I) while the hyper-resorptive phenotype of $Zeb1^{\Delta M/\Delta M}$ osteoclasts could be recapitulated in wild-type osteoclasts by either iii) MtCK1 overexpression alone or iv) the exogenous addition of phosphocreatine (Fig 6A-K; Fig EV4A-D; Appendix Fig S6A-C). Though we agree that Zeb1 can undoubtedly play complex roles in cell biology (Stemmler et al., 2019; Vandewalle et al., 2009), our data are the first to identify the importance of a previously unrecognized Zeb1/MtCK1 axis as an integrator of mitochondrial signaling and osteoclast-dependent bone resorption.

Nevertheless, as suggested by the Reviewer, we performed a series of exploratory studies to address the potential importance of alternate Zeb1 targets in regulating osteoclast activity. First, we find that $Zeb1^{\Delta M/\Delta M}$ osteoclasts display no significant changes in mRNA or protein levels of Nfatc1 (Fig 3B; Appendix Fig S1F and S2A). Indeed, none of the other transcription factors identified in Fig 2B were affected by *Zeb1* targeting. Second, we noted that *Epcam* was also among the top 10 differentially expressed genes in $Zeb1^{\Delta M/\Delta M}$ osteoclasts (Fig 4A and B), and that recent studies indicate that increased EpCAM gene/protein expression can confer a survival advantage and differentiation block in $Zeb1^{-/-}$ hematopoietic stem cells (*J Clin Invest* 131:e129115, 2021). Nevertheless, *Zeb1* deletion did not affect EpCAM protein expression in

osteoclasts though *Epcam* transcription is indeed upregulated (Appendix Fig S4E and F). Expanding on these efforts, we were likewise intrigued by reports that the bone-resorptive capacity of osteoclasts is regulated by the transcriptional co-activator, PGC1β, which controls both mitochondrial biogenesis and the oxidative phosphorylation-mediated cytoskeletal organization critical to sealing zone formation (*J Bone Miner Res* 33:1114, 2018). However, Zeb1 neither regulates *Ppargc1b* gene expression nor mitochondrial biogenesis in osteoclasts (Fig 4A; Appendix Fig S5A-F). Finally, Zeb1 has more recently been reported to regulate cancer cell metabolism via transcriptionally repressing the mitochondrial-localized deacetylase, *Sirt3,* or upregulating transcript levels of the glycolytic enzymes, *Pfkm*, *Hk2*, *Pfkp*, and *Pkm2* (*World J Gastroenterol* 24:4893, 2018; *Theranostics* 11:5926, 2021; *Cell Death Dis* 13:206, 2022). Nevertheless, we likewise excluded *Sirt3*, *Hk2*, *Pkm2*, *Pfkm*, and *Pfkp* as potential Zeb1 targets as well (Appendix Fig S7B). These data have been inserted in the revised text.

3. WM and fluorescent images throughout the text need to be quantified and statistically evaluated.

As suggested by the Reviewer, we have quantified all the western blot and fluorescent images and included statistical analyses. These data have been inserted in the revised text.

Reviewer #2:

General comments:

Based on an unbiased transcriptomic approach, which identified that expression of the transcriptional repressor Zeb1 increases during the course of osteoclastogenesis, the authors generated a mouse model with myeloid-specific Zeb1 inactivation to uncover a previously unknown role of Zeb1 as a negative regulator of bone resorption. Through a comprehensive molecular analysis of Zeb1-deficient osteoclasts they identified increased expression of MtCK1 as a major driver of this phenotype and thereby uncovered a critical role of the creatine kinas axis for osteoclast-mediated bone resorption. All presented data are highly convincing and supported by different methodological approaches, including siRNA-knockdown, promoter binding studies, normalization by treatment with cyclocreatine, and confirmation of the data by experiments with human osteoclasts. The presentation of these novel and relevant data is truly excellent, and there is no need for further improvement. There is only one issue that the authors should consider, i.e. to add a schematic presentation summarizing the Zeb1-dependent mechanism that was uncovered.

We thank the Reviewer for his/her careful review of our work. We have addressed constructive concerns (including a schematic model or graphic abstract) and believe that our new data significantly improves and strengthens our conclusions.

Reviewer #3:

General comments:

In their manuscript, Zhu and colleagues identify the transcriptional repressor Zeb1 as a key factor in regulating the bone-resorbing activity of osteoclasts (OCs) and show that it functions by downregulating mitochondrial creatine kinase (MtCK) and the levels of its product, phosphocreatine. Specifically, the authors identify Zeb1 as a gene that is up-regulated in mature OCs relative to bone marrow-derived macrophages (BMDMs), and that myeloid-specific knockout of Zeb1 in mice reduces bone mass due to increased bone resorption by OCs. The knockout OCs exhibit more prominent actin rings (sealing zones), which are central to resorptive activity, in correlation with increased activity of RhoA in the cells. Of note, differentiation of OCs is unaffected in these mice, as is bone matrix production by osteoblasts. The authors then identify MtCK, but not other forms of creatine kinase, as a target of the transcriptional repressor activity of Zeb1 and establish that MtCK is up-regulated in the Zeb1-knockout OCs, along with increased mitochondrial respiration and ATP production in these cells. These changes are specific since mitochondrial structure, mitochondrial mass, DNA copy number, and levels of key mitochondrial complexes are unchanged. Importantly, these effects, as well as enhanced actin rings, are replicated in OCs that express exogenous MtCK, and also in wild-type OCs exposed to exogenous phosphocreatine. In line with these results, knockdown of MtCK in Zeb1-deficient OCs rescues the mitochondrial abnormalities. Importantly, the findings were replicated also in human monocyte-derived OCs in which expression of Zeb1 was partially down-regulated, while the expected opposite results were obtained upon partial down-regulation of MtCK.

This is a well-conceived study that was performed with care, and contains results that support the conclusions made. The study is presented in a clear manner and adequately discusses relevant literature. Important roles for the cytosolic brain form of creatine kinase in OCs were described previously by Chang et al, 2008 (quoted in the current manuscript), but the authors show that their findings are independent of that form. In all, this manuscript describes a novel role for Zeb1 in regulating OC activity and skeletal structure through regulation of energy metabolism in OCs. OCs are known to require significant amounts of energy for their activity; this study uncovers a novel Zeb1-MtCK-phosphocreatine axis that helps control this key cellular activity, making it relevant to the broader cell biology community.

We thank the Reviewer for his/her careful review of our work. We have addressed each of the constructive concerns below and believe that our new data significantly improves and strengthens our conclusions.

Specific comments:

1. While the Zeb1-MtCK-phosphocreatine axis is well-described, it is less clear how elevated levels of phosphocreatine, which are the product of the axis in the current study, promote the bone-resorbing activity of OCs. Is this driven by enhanced RhoA activity and actin ring formation? Are the RhoA and mitochondrial phenotypes directly connected in this respect? The data also suggest that the energy status of OCs can drive, as opposed to simply enable, their bone resorption activity. How does this fit in with other known mechanisms that regulate OC activity?

Underlining the importance of phosphocreatine in mediating these effects, we found that the addition of exogenous phosphocreatine alone to wild-type osteoclasts significantly increases

GTP-loaded RhoA activity, actin ring formation as well as bone resorbing activity (Fig EV4A-D) in parallel with enhanced mitochondrial respiration and ATP production (Appendix Fig S6A-C). To assess the relative importance of RhoA in controlling osteoclast activation driven by phosphocreatine, cells were treated with a RhoA inhibitor, Y16 (*Proc Natl Acad Sci* 110:3155, 2013). Under these conditions, the enhanced sealing zone formation and bone-resorptive activity observed in phosphocreatine-treated osteoclasts were each significantly downregulated (Appendix Fig S6D and E). Consistent with these observations, while cellular energetics is reported to be strongly correlated with the Rho GTPase activity and cytoskeletal organization in other cell types (Chen et al., 2018; Holmes et al., 2020; Wu et al., 2021; Zhang et al., 2013), the mitochondrial ATP synthase and oxidative-phosphorylation inhibitor, oligomycin A, likewise significantly reduced RhoA activity, sealing zone formation and bone resorption activity of phosphocreatine-treated wild-type osteoclasts (Appendix Fig S6F-H). Taken together, these data support our contention that mitochondrial respiration and RhoA activation are directly connected, acting directly downstream of phosphocreatine-dependent effects on osteoclast activity. These data have been inserted in the revised text.

As indicated by the Reviewer, our results suggest that the energy status of osteoclasts drives bone resorptive activity, a finding consistent with earlier observations that glucose supplementation and mitochondrial respiration mediated by the transcriptional co-activator, PGC1β, the G-protein, Gα13, or the mitochondrial deacetylase, Sirt3, directly control osteoclastic bone-resorbing activity (*J Bone Miner Res* 28:2392, 2013; *J Bone Miner Res* 33:1114, 2018; *Sci Rep* 9:4236, 2019; *JCI Insight* 6:e146728, 2021). We have discussed these points in the revised text.

2. Page 5, 1st paragraph: the authors describe Zeb1 as present in cells that express proteins characteristic of mature OCs. Similarly, Figure 1 compares mature OCs with BMDMs. The authors should examine Zeb1 expression at several time points during the osteoclastogenic process in vitro. When does Zeb1 expression begin? Is MtCK expression correlated with Zeb1 expression throughout the process?

We examined the expression dynamics of Zeb1 and MtCK1 during osteoclastogenic differentiation *in vitro* in a time-dependent manner. We found that both Zeb1 and MtCK1 protein expression are increased during osteoclastogenic differentiation (Fig 3B and 4F; Appendix Fig S2A and S4A). However, under these conditions, Zeb1 expression does not correlate completely with MtCK1 expression. While Zeb1 is expressed as early as day 2, MtCK1 expression is not induced until day 4 (Fig 3B and 4F; Appendix Fig S2A and S4A). It could be speculated that MtCK1 is induced by other unknown transcription factors during osteoclast differentiation, a subject deserving of future study. Independent of our report, similar scenarios have been described wherein Nur77 or Gα13 negatively regulates osteoclast Nfatc1 expression, while Nur77 and Gα13 expression is upregulated together with Nfatc1 during osteoclast differentiation (*Elife* 4:e07217, 2015; *Nat Commun* 8:13700, 2017).

3. The authors show that the actin rings of *Zeb1*-deficient OCs occupy a larger fraction of the cell area (Fig. 3H). Additional data is required for better understanding of this. Is the size (or nuclear number) of Zeb1-deficient OCs changed? Is the number of actin rings per OC altered? Is the alphaVbeta3 integrin altered (localization, phosphorylation) in the mutant OCs?

As requested, we performed quantitative analysis of the number of nuclei per osteoclast, as well as the number of actin rings per osteoclast in *Zeb1*-deficient cells. Our data show that

neither the number of nuclei per osteoclast nor the number of actin rings per osteoclast on bone are altered in *Zeb1*$^{\Delta M/\Delta M}$ osteoclasts (Appendix Fig S2D and E), indicating actin ring size is the dominant phenotypic change observed in *Zeb1*$^{\Delta M/\Delta M}$ osteoclasts.

With regard to integrin signaling, αvβ3 is generally regarded as the major receptor in osteoclasts, mediating signaling cascades linked to tyrosine phosphorylation and activation of c-Src (*J Cell Biol* 152:181, 2001; *J Cell Biol* 176:877, 2007; *J Clin Invest* 107:1137*,* 2001; *J Bone Miner Res* 28:2449, 2013). As requested, to further determine αvβ3 localization and activation status in *Zeb1*-deleted osteoclasts, we assessed its membrane localization and downstream c-Src phosphorylation in wild-type vs *Zeb1*$^{\Delta M/\Delta M}$ osteoclasts. However, we did not detect changes of surface β3 integrin levels or vitronectin-triggered c-Src phosphorylation in *Zeb1*$^{\Delta M/\Delta M}$ osteoclasts (Appendix Fig S2F-H). These data have been inserted in the revised text.

Minor points:

1) The genetic background(s) of the mice used should be specified. Also, the status of the Zeb1 and Cre alleles of the of the littermate "wild-type" control mice (Figure 2) should be defined.

The genetic backgrounds of the mice are listed in the “Materials and Methods” section. *Zeb1*$^{+/+}$ mice are defined as “wild-type” control while *Zeb1*$^{f/+}$ and *Zeb*$^{f/f}$ mice, which uniformly display normal bone and osteoclast phenotypes, are described as such in the text and are considered ‘control’ mice. This information has been inserted in the revised text.

2) The nature of the catalytically-inactive MtCK C316G mutant should be noted (also) in the main text.

The metabolic inactive MtCK1^{C316G} mutant represents the mutation of the cysteine residue C316, which is required for synergistic binding of MtCK1 to its substrate, into glycine (*Nat Med* 23:301, 2017; *Biochemistry* 32:7022,1993). As requested, we have added the information of catalytically-inactive MtCK1 C316G mutants (*Nat Med* 23:301, 2017; *Biochemistry* 32:7022,1993) in the main text as well.

3) Legends to Figure 1 and Supp. Figure 1: There is some discrepancy between the panel indicators in the legend texts and in the figures.

Corrected.

4) Figure 2B and Supp. Figure 2B: To which bone type does the data refer?

As shown in the corresponding figure legends, Fig 2B refers to the quantification of bone phenotype for 3-month-old wild-type and *Zeb1*$^{\Delta M/\Delta M}$ male mice, while Fig EV2B (previous Suppl Fig 2B) refers to the quantitative analysis of bone phenotype for 3-month-old wild-type and *Zeb1*$^{\Delta M/\Delta M}$ female mice.

5) Figure 6C: MtCK Western blot: assuming that the lower band represents the exogenous protein, why does it migrate differently from the endogenous one?

We found that endogenous MtCK1 in mouse osteoclasts always displays two bands, presumably due to the fact that MtCK1 protein can be expressed as either of two isoforms

produced as a consequence of alternative splicing with molecular weights of 47 kDa and 50 kDa, respectively (*Biochim Biophys Acta.* 1833:2844, 2013). Therefore, we would infer that the double bands of MtCK1 in Fig 6C represent two isoforms due to alternative splicing, which is similar with MtCK1 bands in some other cell types (*Biochim Biophys Acta.* 1833:2844, 2013; *Nat Med* 23:301, 2017).

6) Enhanced expression of MtCK in human monocyte-derived OCs should be demonstrated also by protein blotting and not only by comparing immunofluorescence images (Supp. Fig. 4).

Enhanced expression of MtCK1 in si*Zeb1*-targeted human monocyte-derived osteoclasts was demonstrated by both western blot (Fig EV 3E and F) and immunofluorescence (Fig EV 3G and H).

7) Supp. Figure 5: mature OCs are large fragile cells that might not do well in FACS analysis. Panel A shows images of mature OCs, while the Methods section (p. 19) describes use of OC precursors in this study. Can the authors clarify?

To clarify, panel A of Appendix Fig S5 (previous Suppl Fig 5) refers to immunofluorescence of BMDMs and osteoclasts cultured on glass generated from wild-type or *Zeb1*$^{\Delta M/\Delta M}$ mice, while panel B of Appendix Fig S5 (previous Suppl Fig 5) refers to flow cytometry analysis of detached BMDMs and osteoclasts differentiated on petri dishes generated from wild-type or *Zeb1*$^{\Delta M/\Delta M}$ mice. As the Reviewer correctly noted, mature OCs plated on tissue culture plastic are fragile and not suitable for FACS analysis. Consequently, we used petri dishes as the culture surface because osteoclasts generated on this surface are smaller and more readily detached for flow cytometry (*J Bone Miner Res* 31:1899, 2016; *J Clin Invest* 127:2555, 2017). We have added the corresponding details to the text.

8) P.10, middle paragraph, speaks of "RhoA levels" - this may be misleading since it refers to RhoA activity or levels of active RhoA.

Agreed. We have corrected references in the text to “RhoA activity”.

References cited in the Response to Reviewers (Alphabetical order)

Almotiri A, Alzahrani H, Menendez-Gonzalez JB, Abdelfattah A, Alotaibi B, Saleh L, Greene A, Georgiou M, Gibbs A, Alsayari A, Taha S, Thomas LA, Shah D, Edkins S, Giles P, Stemmler MP, Brabletz S, Brabletz T, Boyd AS, Siebzehnrubl FA, Rodrigues NP. Zeb1 modulates hematopoietic stem cell fates required for suppressing acute myeloid leukemia. *J Clin Invest.* 2021; 131(1):e129115.

Bae S, Lee MJ, Mun SH, Giannopoulou EG, Yong-Gonzalez V, Cross JR, Murata K, Giguère V, van der Meulen M, Park-Min KH. MYC-dependent oxidative metabolism regulates osteoclastogenesis via nuclear receptor ERRα. *J Clin Invest.* 2017; 127(7):2555-2568.

Datler C, Grimm S. Reconstitution of CKMT1 expression fails to rescue cells from mitochondrial membrane potential dissipation: implications for controlling RNAi experiments. *Biochim Biophys Acta.* 2013; 1833(12):2844-2855.

Feng X, Novack DV, Faccio R, Ory DS, Aya K, Boyer MI, McHugh KP, Ross FP, Teitelbaum SL. A Glanzmann's mutation in beta 3 integrin specifically impairs osteoclast function. *J Clin Invest.* 2001; 107(9):1137-1144.

Fenouille N, Bassil CF, Ben-Sahra I, Benajiba L, Alexe G, Ramos A, Pikman Y, Conway AS, Burgess MR, Li Q, Luciano F, Auberger P, Galinsky I, DeAngelo DJ, Stone RM, Zhang Y, Perkins AS, Shannon K, Hemann MT, Puissant A, Stegmaier K. The creatine kinase pathway is a metabolic vulnerability in EVI1-positive acute myeloid leukemia. *Nat Med.* 2017; 23(3):301-313.

Furter R, Furter-Graves EM, Wallimann T. Creatine kinase: the reactive cysteine is required for synergism but is nonessential for catalysis. *Biochemistry.* 1993; 32(27):7022-7029.

Goettsch C, Babelova A, Trummer O, Erben RG, Rauner M, Rammelt S, Weissmann N, Weinberger V, Benkhoff S, Kampschulte M, Obermayer-Pietsch B, Hofbauer LC, Brandes RP, Schröder K. NADPH oxidase 4 limits bone mass by promoting osteoclastogenesis. *J Clin Invest.* 2013; 123(11):4731-4738.

Han X, Duan X, Liu Z, Long Y, Liu C, Zhou J, Li N, Qin J, Wang Y. ZEB1 directly inhibits GPX4 transcription contributing to ROS accumulation in breast cancer cells. *Breast Cancer Res Treat.* 2021; 188(2):329-342.

Han X, Long Y, Duan X, Liu Z, Hu X, Zhou J, Li N, Wang Y, Qin J. ZEB1 induces ROS generation through directly promoting MCT4 transcription to facilitate breast cancer. *Exp Cell Res.* 2022; 412(2):113044.

Ibáñez L, Abou-Ezzi G, Ciucci T, Amiot V, Belaïd N, Obino D, Mansour A, Rouleau M, Wakkach A, Blin-Wakkach C. Inflammatory Osteoclasts Prime TNFα-Producing CD4+ T Cells and Express CX3CR1. *J Bone Miner Res.* 2016; 31(10):1899-1908.

Indo Y, Takeshita S, Ishii KA, Hoshii T, Aburatani H, Hirao A, Ikeda K. Metabolic regulation of osteoclast differentiation and function. *J Bone Miner Res.* 2013; 28(11):2392-2399.

Jiang H, Wei H, Wang H, Wang Z, Li J, Ou Y, Xiao X, Wang W, Chang A, Sun W, Zhao L, Yang S. Zeb1-induced metabolic reprogramming of glycolysis is essential for macrophage polarization in breast cancer. *Cell Death Dis.* 2022; 13(3):206.

Kang IS, Kim C. NADPH oxidase gp91phox contributes to RANKL-induced osteoclast differentiation by upregulating NFATc1. *Sci Rep.* 2016; 6:38014.

Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, Kim N, Lee SY. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood.* 2005; 106(3):852-859.

Li X, Wei W, Huynh H, Zuo H, Wang X, Wan Y. Nur77 prevents excessive osteoclastogenesis by inducing ubiquitin ligase Cbl-b to mediate NFATc1 self-limitation. *Elife.* 2015;4:e07217.

Ling W, Krager K, Richardson KK, Warren AD, Ponte F, Aykin-Burns N, Manolagas SC, Almeida M, Kim HN. Mitochondrial Sirt3 contributes to the bone loss caused by aging or estrogen deficiency. *JCI Insight.* 2021; 6(10):e146728.

Nagai Y, Osawa K, Fukushima H, Tamura Y, Aoki K, Ohya K, Yasuda H, Hikiji H, Takahashi M, Seta Y, Seo S, Kurokawa M, Kato S, Honda H, Nakamura I, Maki K, Jimi E. p130Cas, Crk-associated substrate, plays important roles in osteoclastic bone resorption. *J Bone Miner Res.* 2013; 28(12):2449-2462.

Nakano S, Inoue K, Xu C, Deng Z, Syrovatkina V, Vitone G, Zhao L, Huang XY, Zhao B. G-protein Gα13 functions as a cytoskeletal and mitochondrial regulator to restrain osteoclast function. *Sci Rep.* 2019; 9(1):4236.

Sanjay A, Houghton A, Neff L, et al. Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. *J Cell Biol.* 2001; 152(1):181-195.

Schröder K. NADPH oxidases in bone homeostasis and osteoporosis. *Free Radic Biol Med.* 2019; 132:67-72.

Shang X, Marchioni F, Evelyn CR, Sipes N, Zhou X, Seibel W, Wortman M, Zheng Y. Small-molecule inhibitors targeting G-protein-coupled Rho guanine nucleotide exchange factors. *Proc Natl Acad Sci U S A.* 2013; 110(8):3155-3160.

Stemmler MP, Eccles RL, Brabletz S, Brabletz T. Non-redundant functions of EMT transcription factors. *Nat Cell Biol.* 2019; 21(1):102-112.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell.* 2002; 3(6):889-901.

Vandewalle C, Van Roy F, Berx G. The role of the ZEB family of transcription factors in development and disease. *Cell Mol Life Sci.* 2009; 66(5):773-787.

Wu M, Chen W, Lu Y, Zhu G, Hao L, Li YP. Gα13 negatively controls osteoclastogenesis through inhibition of the Akt-GSK3β-NFATc1 signalling pathway. *Nat Commun.* 2017; 8:13700.

Xu WY, Hu QS, Qin Y, Zhang B, Liu WS, Ni QX, Xu J, Yu XJ. Zinc finger E-box-binding homeobox 1 mediates aerobic glycolysis via suppression of sirtuin 3 in pancreatic cancer. *World J Gastroenterol.* 2018; 24(43):4893-4905.

Zhang Y, Rohatgi N, Veis DJ, Schilling J, Teitelbaum SL, Zou W. PGC1β Organizes the Osteoclast Cytoskeleton by Mitochondrial Biogenesis and Activation. *J Bone Miner Res.* 2018; 33(6):1114-1125.

Zhou Y, Lin F, Wan T, Chen A, Wang H, Jiang B, Zhao W, Liao S, Wang S, Li G, Xu Z, Wang J, Zhang J, Ma H, Lin D, Li Q. ZEB1 enhances Warburg effect to facilitate tumorigenesis and metastasis of HCC by transcriptionally activating PFKM. *Theranostics.* 2021; 11(12):5926-5938.

Zou W, Kitaura H, Reeve J, et al. Syk, c-Src, the alphavbeta3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption. *J Cell Biol.* 2007;176(6):877-888.

Dear Dr Weiss,

Thank you for submitting your study, "A Zeb1/MtCK1 Metabolic Axis Controls Osteoclast Activation and Skeletal Remodeling", to EMBO Journal. Your revised version has now been reviewed once again by the three referees who saw your initial submission. I have attached their reports to the bottom of this email. I have read the reports and your revised manuscript very carefully and have discussed them with my editorial colleagues. As you will see, the reports are not unanimously positive. I remain concerned over the relative significance of Zeb1 for MtCK induction explained in the comments of referee 3. Therefore at this stage, I am not able to pursue this manuscript towards publication.

I appreciate that, at its core, your work investigates the relationship between ZEB1 and MtCK1 during osteoclast differentiation. I acknowledge that Referee 2 was very impressed by your work; Referees 2 and 3 also comment positively that your revised manuscript has been significantly improved. However, I share their concerns that your conclusions are still not fully supported by key aspects of the data you present.

I am verry sorry that it is not possible for me to be more positive as your study clearly contains many interesting aspects. I also hope that you will continue to see The EMBO Journal as a suitable platform for your future work.

Best wishes,

William Teale

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

****

Referee #1:

The authors performed additional experiments and address some of the reviewers comments and suggestions. The manuscript is improved but some concerns still remain.
As already mentioned in the first review round, it is very surprising that ZEB1 acts, more or less, only via one protein i.e. the mitochondrial creatine kinase 1. And this is despite the indications from experimental data and published literature that other genes/proteins and transcription factors may be involved.
In their point-by-point reply the authors simply eliminate all alternatives by performing ROS measurements (with a kit that measures H2O2 only in the extracellular space in a cell population) and gene/protein expression analyses. By doing this, however, they indirectly question findings from several previous studies. A more careful approach including a more robust and detailed experimental validation could have been helpful when making such claims.

Referee #2:

Whereas I did not request specific modifications of this turly impressvie manuscript (besides suggesting to include a schematic presentatin of the main conclusions), the authors have further improved their manuscript by addressing the critical comments of the other reviewers.

Referee #3:

The authors have responded to my comments in a satisfactory manner, except for the following points:

Major point 2 (Expression of Zeb1 and MtCK during osteoclastogenesis, and are they temporally correlated):

The authors provide protein blots that show that MtCK is induced two days later in osteoclastogenesis than Zeb1. The explanation provided for this time gap in the rebuttal letter (but apparently not in the manuscript) is that "it could be speculated that MtCK is induced by other unknown transcription factors during osteoclast differentiation", and that this issue requires further study. This comment, which literally means that Zeb1 does not induce MtCK, contradicts a central conclusion of the current manuscript and needs to be addressed. The authors should note the existence of the temporal difference in induction of Zeb1

vs. MtCK proteins in the text and suggest explanations. These may include also involvement of transcription factors IN ADDITION TO Zeb1, and modifications in the profile of genes regulated by Zeb1 due to the changing cellular environment during osteoclast production.

Major point 3: (size of Zeb1-deficient OCs and their actin rings):

Please explain the nature of the data in Fig. S2E - number of actin rings/OC. The statement "Data are representative of at least 3 independent experiments with biological replicates" is not clear in this context. The six data points cannot be from representative cells (number of rings per individual cell is a whole number), so they apparently represent averages, but of what/how many cells?

Minor point 4: The question was what type of bone was analyzed in the original Fig 2B and Supp Fig. 2b. Was it the distal femur as in some of the other panels in the figures?

We would like to bring several issues to your attention.

Reviewer #1 asks, to paraphrase, “How is it possible that Zeb1 targets but a single molecule to exert such effects on osteoclast function?” But, our work more specifically asks “How does Zeb1 regulate osteoclast bone resorption *in vitro* and *in vivo*?” While we agree that the findings are surprising, our proofs are hardly limited to the fact that MtCK1 was the most highly unregulated transcript identified in the knockout cells. While the upregulation of MtCK1 transcript and protein levels provided a tantalizing hint, it was only a hint. Most importantly, the Reviewer appears to have *completely* overlooked the fact that our identification of MtCK1 as the critical Zeb1 target in osteoclasts was/is based on the fact that the hyperactive bone resorptive phenotype observed in *Zeb1 *knockout cells could be normalized *completely* following either i) MtCK1 knockdown to levels similar to that found in wild-type osteoclasts or ii) the addition of the creatine kinase inhibitor, cyclocreatine. If Zeb1 exerted other effects on osteoclast function, how was normal bone resorption restored in Zeb1 knockout cells by targeting MtCK1 activity alone? Conversely, the hyper-resorptive phenotype of *Zeb1 *knockout osteoclasts was recapitulated fully in wild-type osteoclasts by either i) overexpressing MtCK1 alone or ii) increasing phosphocreatine levels – without affecting Zeb1 expression. We agree that Zeb1 undoubtedly plays complex roles in other cell populations, but our data clearly demonstrate that the osteoclast hyper-resorptive phenotype observed in Zeb1 knockouts – either *in vitro* or in* vivo, *can be ascribed solely to MtCK1 targeting. If the Reviewer has an alternate explanations for these data, we would welcome the insight.

As an aside, the comments regarding hydrogen peroxide measurements are simply erroneous. Hydrogen peroxide has a cell permeability coefficient similar to water and does not ‘accumulate’ in the intracellular compartments. I worked in the field of reactive oxygen metabolites for the first 25 years of my career and have published extensively in this area in the likes of *Science*, *New Engl J Med*, *JCI*, *JBC* and the* PNAS*. My background notwithstanding, there is no data in the literature linking oxidative metabolism to osteoclast bone resorption. Prior reports are limited to effects of oxygen metabolites on osteoclastogenesis, which is completely unaffected in our studies – again, *in vitro* as well as *in vivo*. Further, we find no effect of Zeb1 targeting on *any* of the oxidases known to be expressed in osteoclasts. Our findings in this regard do not ‘contradict’ any of the existing literature in the field. I would add that while many attempt to monitor reactive oxygen metabolites with intracellular fluorescent dyes, cognoscenti in the field are well aware that virtually all of these assay systems are non-specific (e.g., *Arch* *Biochem Biophys* 617:38, 2017; *Nat Metab* 4:651, 2022).

Reviewer 3’s comments are more enigmatic/problematic to us and suggest a serious misinterpretation of our work. In their original review, we were asked;* “The authors* *should examine Zeb1 expression at several time points during the osteoclastogenic process in vitro.* *When does Zeb1 expression begin? Is MtCK expression correlated with Zeb1 expression throughout* *the process?” *We provided this information and demonstrated that Zeb1 protein levels are detected as early as day 2 of the osteoclastogenic program while MtCK1 begins to be expressed at day 4. As we predicted – and entirely consistent with our central hypothesis, in the absence of *Zeb1*, MtCK1 is expressed at even higher levels (i.e., we identified Zeb1 as an *MtCK1* repressor). Additional data were also provided in the revised manuscript demonstrating that placing wild-type osteoclasts on bone to initiate active resorption triggered a predicted *decrease* in endogenous Zeb1 levels coincident with the expected *increase* in MtCK1 levels. We pointed out in our prior response, as an aside, that the transcription factors responsible for

inducing MtCK1 mRNA are unknown, which is true, but certainly outside the confines of our report. We only stated for the Reviewer's interest that '...it could be speculated that MtCK1 is induced by other unknown transcription factors during osteoclast differentiation.' However, the Reviewer retorted,* "This comment, which literally means that Zeb1 does not induce MtCK1, contradicts a central conclusion of the current manuscript and needs to be addressed." *I really don't have a politic way of responding to this comment other than this is a complete misreading of our work. Zeb1 does not *induce* MtCK1, it is a transcriptional repressor that downregulates MtCK1. *All* of our data support this conclusion.

Given these concerns, I am wondering if this decision might be revisited. We would happily revise the final text to insure that others do not misinterpret our work (though we note that issues raised by Reviewer #1 were not identified by Reviewer #2 or #3 and likewise, the critique offered by Reviewer #3 was not shared by the first two reviewers).

Dear Steve,

Thank you once again for trusting the editorial office at EMBO Journal, and appealing against our decision on your recent manuscript (EMBOJ-2022-111148). We judged your arguments to be sufficiently substantive to seek an adjudicating opinion from a fourth reviewer. This reviewer was given access to all correspondence, referee reports and manuscript files. I am happy to say that, as you will see from the brief report at the bottom of this email, their decision was to support fully the publication of your work. I would therefore like to invite you to submit a revised version of the manuscript which includes a discussion of the points raised by reviewers 1 and 3 in their second referee reports (including a point-by-point response detailing the changes made), and addresses some minor editorial points. In this regard would you please:

- remove coloured text from the final text file (.doc)
- check the data referred to in the Data Availability Statement is present and accessible
- rename the conflict of interest statement as the "Disclosure and Competing Interests Statement"
- remove the AC/CrediT section from the text
- add reference to grant number P30-AR069620 on our submission webpage
- include page numbers to the table of contents in Appendix 1
- arrange the Source Data into one zipped file per figure
- remove the synopsis text and image from the main text and include them as separate files.

EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Use the link below to submit your revision:

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

Referee #4:

As an additional reviewer, I have carefully read the revised manuscript and the authors' responses. In my view, the authors have satisfied the main comments brought up by the reviewers. This paper sheds insight on a new mode of metabolic regulation by Zeb1 in osteoclasts and is suitable for publication.

Responses to Reviewers

EMBOJ-2022-111148R2: "A Zeb1/Mitochondrial Creatine Kinase Metabolic Axis Controls Osteoclast Activation and Skeletal Remodeling" by Lingxin Zhu, Yi Tang, Xiao-Yan Li, Samuel A. Kerk, Wenqing Feng, Xiaoyue Sun, Geoffrey E. Hespe, Zijun Wang, Jingwen Yang, Jun Ma, Jung-Sun Cho, Marc P. Stemmler, Simone Brabletz, Thomas Brabletz, Evan T. Keller, Costas A. Lyssiotis, and Stephen J. Weiss

Reply to Editor's comments:

General comments:

We judged your arguments to be sufficiently substantive to seek an adjudicating opinion from a fourth reviewer. This reviewer was given access to all correspondence, referee reports and manuscript files. I am happy to say that, as you will see from the brief report at the bottom of this email, their decision was to support fully the publication of your work. I would therefore like to invite you to submit a revised version of the manuscript which includes a discussion of the points raised by reviewers 1 and 3 in their second referee reports (including a point-by-point response detailing the changes made), and addresses some minor editorial points.

We appreciate the positive feedback and have now addressed all remaining concerns as well as format requirements. We believe our manuscript has been further improved, thereby ensuring that the work is interpreted correctly. Our point-by-point responses are included below.

Specific points:

- remove coloured text from the final text file (.doc)

Done.

- check the data referred to in the Data Availability Statement is present and accessible

Done.

- rename the conflict of interest statement as the "Disclosure and Competing Interests Statement"

Done.

- remove the AC/CrediT section from the text

Deleted.

- add reference to grant number P30-AR069620 on our submission webpage

Reference to this grant number has been added on the submission webpage.

- include page numbers to the table of contents in Appendix 1

Done.

- arrange the Source Data into one zipped file per figure

Done.

- remove the synopsis text and image from the main text and include them as separate files.

Done.

Reviewer #1:

General comments:

The authors performed additional experiments and address some of the reviewers comments and suggestions. The manuscript is improved but some concerns still remain.

We thank reviewer for the overall positive evaluation of our manuscript. We have carefully addressed each of the concerns raised and include further descriptions of works outlining the complex roles played by Zeb1 in other cell populations, which we believe only strengthens our conclusions.

Specific comments:

1. As already mentioned in the first review round, it is very surprising that ZEB1 acts, more or less, only via one protein i.e. the mitochondrial creatine kinase 1. And this is despite the indications from experimental data and published literature that other genes/proteins and transcription factors may be involved.

Reviewer #1 asks, to paraphrase, "How is it possible that Zeb1 targets but a single molecule to exert such effects on osteoclast function?". However, our work more specifically addresses the question, "How does Zeb1 play such a major role in regulating osteoclast bone resorption *in vitro* and *in vivo*?" While we agree that the findings are surprising, our proofs are hardly limited to the fact that mitochondrial creatine kinase 1 (MtCK1), an enzyme critical to mitochondrial oxidative activity and energy metabolism (*Biochem J* 281:21, 1992; *Biochim Biophys Acta* 1762:164, 2006; *Amino Acids* 40:1271, 2011), was the most highly upregulated transcript identified in the knockout cells (Fig 4A and B). Most importantly, our identification of the MtCK1-mediated phosphocreatine energy shuttle as the critical Zeb1 target in osteoclasts was/is based on the fact that the hyperactive bone resorptive phenotype observed in *Zeb1* knockout cells could be normalized completely following either i) MtCK1 knockdown to levels similar to those found in wild-type osteoclasts or ii) the direct inhibition of their heightened creatine kinase activity with cyclocreatine (Fig EV4E-K and 5I). Conversely, the hyper-resorptive phenotype of *Zeb1* knockout osteoclasts was recapitulated fully in wild-type osteoclasts by either i) overexpressing MtCK1 alone or ii) increasing phosphocreatine levels – without affecting Zeb1 expression (Fig 6A-K; Fig EV4A-D; Appendix Fig S6A-C). We agree that Zeb1 undoubtedly plays complex roles in other cell populations (*Nat Cell Biol* 21:102, 2019; *Cell Mol Life Sci* 66:773, 2009; *Nat Med* 23:568, 2017; *Nat Cell Biol* 19:518, 2017; *EMBO J* 39:e103209, 2020; *J Clin Invest* 131:e129115, 2021), but our data clearly demonstrate that the hyper-resorptive phenotype of *Zeb1* knockout osteoclasts – either *in vitro* or *in vivo*, can be ascribed largely, if not completely, to MtCK1 targeting and mitochondrial energy metabolism. Nevertheless, we have expanded the Discussion to include the distinct roles played by Zeb1 in other cell populations.

2. In their point-by-point reply, the authors simply eliminate all alternatives by performing ROS measurements (with a kit that measures H_2O_2 only in the extracellular space in a cell population) and gene/protein expression analyses. By doing this, however, they indirectly question findings from several previous studies. A more careful approach including a more robust and detailed experimental validation could have been helpful when making such claims.

With all due respect, we disagree with this assessment. The role of Zeb1 in regulating ROS generation is not only complex, but also cell- as well as context-dependent (*Nat Med* 23:568, 2017; *Gut* 68:2129, 2019; *Breast Cancer Res Treat* 188:329, 2021; *Exp Cell Res* 412:113044, 2022; *Cell Death Dis* 13:735, 2022). As requested in the original review, we carefully monitored ROS levels in wild-type vs knockout osteoclasts using both the intracellular ROS probe, DCFH-DA, and a sensitive peroxidase-dependent assay for H_2O_2 (*Arch Biochem Biophys* 617:38, 2017; *Nat Metab* 4:651, 2022). Using either system, we reported that ROS levels are comparable between wild-type and *Zeb1*$^{\Delta M/\Delta M}$ osteoclasts (Appendix Fig S4G and H). Further, we find no effect of Zeb1 targeting on any of the ROS-linked oxidases known to be expressed in osteoclasts (Fig 4A; Appendix Fig S4I). In this regard, it is important to note that prior reports describing effects of reactive oxygen metabolites on osteoclast function are limited to osteoclastogenesis, not bone resorption per se (*Blood* 106:852, 2005; *J Biol Chem* 285:6913, 2010; *J Clin Invest* 123:4731, 2013; *Nat Commun* 5:3773, 2014; *J Bone Miner Metab* 33:359; 2015; *Sci Rep* 6:38014, 2016; *Free Radic Biol Med* 132:67, 2019). In our system, osteoclast differentiation is completely unaffected either *in vitro* or *in vivo* (Fig 2C and D, 3A and B; Appendix Fig S1F and S2A), a finding consistent with our conclusion that myeloid-specific depletion of *Zeb1* does not affect osteoclast ROS formation. Finally, we are unaware of literature that unequivocally documents a role for osteoclast-derived ROS in bone resorption directly. Hence, we are not in agreement with the conclusion that our findings 'contradict' literature relevant to the field.

Reviewer #2:

General comments:

Whereas I did not request specific modifications of this truly impressive manuscript (besides suggesting to include a schematic presentation of the main conclusions), the authors have further improved their manuscript by addressing the critical comments of the other reviewers.

Thank you for the positive evaluation of our manuscript.

Reviewer #3:

General comments:

The authors have responded to my comments in a satisfactory manner, except for the following points:

Specific comments:

1. Major point 2 (Expression of Zeb1 and MtCK during osteoclastogenesis, and are they temporally correlated): The authors provide protein blots that show that MtCK is induced two days later in osteoclastogenesis than Zeb1. The explanation provided for this time gap in the rebuttal letter (but apparently not in the manuscript) is that "it could be speculated that MtCK is induced by other unknown transcription factors during osteoclast differentiation", and that this issue requires further study. This comment, which literally means that Zeb1 does not induce MtCK, contradicts a central conclusion of the current manuscript and needs to be addressed. The authors should note the existence of the temporal difference in induction of Zeb1 vs. MtCK proteins in the text and suggest explanations. These may include also involvement of transcription factors IN ADDITION TO Zeb1, and modifications in the profile of genes regulated by Zeb1 due to the changing cellular environment during osteoclast production.

We regret that there is a misunderstanding here, i.e., Zeb1 does not *induce* MtCK1, but instead acts as a transcriptional repressor of the kinase. We previously demonstrated that Zeb1 protein levels are detected by day 2 of the osteoclastogenic program while MtCK1 begins to be expressed at day 4 (Fig 3B and 4F; Appendix Fig S2A and S4A), a time point coincident with the appearance of mature, functionally active, bone-resorbing osteoclasts critical to skeletal remodeling (*Nature* 423:337, 2003; *Nat Rev Genet* 4:638, 2003). As we predicted – and entirely consistent with our central hypothesis, in the absence of Zeb1, MtCK1 is expressed at even higher levels in mature osteoclasts both *in vitro* and *in vivo* (Fig 4E-H; Fig EV3D; Appendix Fig S4A-D). Additional data were also provided in the revised manuscript demonstrating that culturing wild-type osteoclasts atop bone to initiate active resorption triggered a predicted decrease in endogenous Zeb1 levels coincident with the expected increase in MtCK1 levels (Appendix Fig S8A-D). That having been said, the transcription factor(s) responsible for inducing *Ckmt1* mRNA are presently unknown, but we believe that the identification of these factors lies outside the confines of our current report. We only stated for the Reviewer's interest that '...it could be speculated that MtCK1 is induced by other unknown transcription factors during osteoclast differentiation'.

2. Major point 3: (size of Zeb1-deficient OCs and their actin rings):

Please explain the nature of the data in Fig. S2E - number of actin rings/OC. The statement "Data are representative of at least 3 independent experiments with biological replicates" is not clear in this context. The six data points cannot be from representative cells (number of rings per individual cell is a whole number), so they apparently represent averages, but of what/how many cells?

With respect to the data presented in Appendix Fig. S2E, the six data points represent six average numbers derived from six independent experiments (n = 6). Each data point is the average of 2 technical replicates from one independent experiment, while 3 random fields from each technical replicate are counted for number of actin rings per osteoclast. As each field includes approximately 4 osteoclasts. As such, each biological replicate involves the counting of

no fewer than 24 osteoclasts. We have added the exact biological replicate number in the Figure legend to make these results more clear.

Minor points:

1) Minor point 4: The question was what type of bone was analyzed in the original Fig 2B and Supp Fig. 2b. Was it the distal femur as in some of the other panels in the figures?

Male as well as female femur trabecular bone were analyzed in Figure 2B and Suppl Figure 2b (now Fig EV2B), respectively. For the other panels in Figure 2, male distal femur was analyzed with nanoCT saggital section/3D reconstruction, HE, TRAP, Goldner's trichrome staining, and double-calcein bone labeling (Fig 2A and C-F). For panel C of Figure EV2, the cortical thickness of the midsection of male femur bone was analyzed by nanoCT, while panel D and E of Figure EV2 are nanoCT results of male vertebral bone. This information has been inserted in the revised text.

Reviewer #4:

General comments:

As an additional reviewer, I have carefully read the revised manuscript and the authors' responses. In my view, the authors have satisfied the main comments brought up by the reviewers. This paper sheds insight on a new mode of metabolic regulation by Zeb1 in osteoclasts and is suitable for publication.

Thank you for the positive evaluation of our manuscript.

References cited in the Response to Reviewers (Alphabetical order)

Almotiri A, Alzahrani H, Menendez-Gonzalez JB, Abdelfattah A, Alotaibi B, Saleh L, Greene A, Georgiou M, Gibbs A, Alsayari A, Taha S, Thomas LA, Shah D, Edkins S, Giles P, Stemmler MP, Brabletz S, Brabletz T, Boyd AS, Siebzehnrubl FA, Rodrigues NP. Zeb1 modulates hematopoietic stem cell fates required for suppressing acute myeloid leukemia. *J Clin Invest.* 2021;131(1):e129115.

Bartell SM, Kim HN, Ambrogini E, Han L, Iyer S, Serra Ucer S, Rabinovitch P, Jilka RL, Weinstein RS, Zhao H, O'Brien CA, Manolagas SC, Almeida M. FoxO proteins restrain osteoclastogenesis and bone resorption by attenuating H2O2 accumulation. *Nat Commun.* 2014;5:3773.

Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature.* 2003;423(6937):337-342.

Callaway DA, Jiang JX. Reactive oxygen species and oxidative stress in osteoclastogenesis, skeletal aging and bone diseases. *J Bone Miner Metab.* 2015;33(4):359-370.

de Barrios O, Sanchez-Moral L, Cortés M, Ninfali C, Profitós-Pelejà N, Martínez-Campanario MC, Siles L, Del Campo R, Fernández-Aceñero MJ, Darling DS, Castells A, Maurel J, Salas A, Dean DC, Postigo A. ZEB1 promotes inflammation and progression towards inflammation-driven carcinoma through repression of the DNA repair glycosylase MPG in epithelial cells. *Gut.* 2019;68(12):2129-2141.

Feldker N, Ferrazzi F, Schuhwerk H, Widholz SA, Guenther K, Frisch I, Jakob K, Kleemann J, Riegel D, Bönisch U, Lukassen S, Eccles RL, Schmidl C, Stemmler MP, Brabletz T, Brabletz S. Genome-wide cooperation of EMT transcription factor ZEB1 with YAP and AP-1 in breast cancer. *EMBO J.* 2020;39(17):e103209.

Goettsch C, Babelova A, Trummer O, Erben RG, Rauner M, Rammelt S, Weissmann N, Weinberger V, Benkhoff S, Kampschulte M, Obermayer-Pietsch B, Hofbauer LC, Brandes RP, Schröder K. NADPH oxidase 4 limits bone mass by promoting osteoclastogenesis. *J Clin Invest.* 2013;123(11):4731-4738.

Han X, Duan X, Liu Z, Long Y, Liu C, Zhou J, Li N, Qin J, Wang Y. ZEB1 directly inhibits GPX4 transcription contributing to ROS accumulation in breast cancer cells. *Breast Cancer Res Treat.* 2021;188(2):329-342.

Han X, Long Y, Duan X, Liu Z, Hu X, Zhou J, Li N, Wang Y, Qin J. ZEB1 induces ROS generation through directly promoting MCT4 transcription to facilitate breast cancer. *Exp Cell Res.* 2022;412(2):113044.

Kalyanaraman B, Hardy M, Podsiadly R, Cheng G, Zielonka J. Recent developments in detection of superoxide radical anion and hydrogen peroxide: Opportunities, challenges, and implications in redox signaling. *Arch Biochem Biophys.* 2017;617:38-47.

Kang IS, Kim C. NADPH oxidase gp91phox contributes to RANKL-induced osteoclast differentiation by upregulating NFATc1. *Sci Rep.* 2016;6:38014.

Kim MS, Yang YM, Son A, Tian YS, Lee SI, Kang SW, Muallem S, Shin DM. RANKL-mediated reactive oxygen species pathway that induces long lasting Ca2+ oscillations essential for osteoclastogenesis. *J Biol Chem.* 2010;285(10):6913-6921.

Krebs AM, Mitschke J, Lasierra Losada M, Schmalhofer O, Boerries M, Busch H, Boettcher M, Mougiakakos D, Reichardt W, Bronsert P, Brunton VG, Pilarsky C, Winkler TH, Brabletz S, Stemmler MP, Brabletz T. The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer. *Nat Cell Biol.* 2017;19(5):518-529.

Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, Kim N, Lee SY. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood.* 2005;106(3):852-859.

Morel AP, Ginestier C, Pommier RM, Cabaud O, Ruiz E, Wicinski J, Devouassoux-Shisheboran M, Combaret V, Finetti P, Chassot C, Pinatel C, Fauvet F, Saintigny P, Thomas E, Moyret-Lalle C, Lachuer J, Despras E, Jauffret JL, Bertucci F, Guitton J, Wierinckx A, Wang Q, Radosevic-Robin N, Penault-Llorca F, Cox DG, Hollande F, Ansieau S, Caramel J, Birnbaum D, Vigneron AM, Tissier A, Charafe-Jauffret E, Puisieux A. A stemness-related ZEB1-MSRB3 axis governs cellular pliancy and breast cancer genome stability. *Nat Med.* 2017;23(5):568-578.

Murphy MP, Bayir H, Belousov V, Chang CJ, Davies KJA, Davies MJ, Dick TP, Finkel T, Forman HJ, Janssen-Heininger Y, Gems D, Kagan VE, Kalyanaraman B, Larsson NG, Milne GL, Nyström T, Poulsen HE, Radi R, Van Remmen H, Schumacker PT, Thornalley PJ, Toyokuni S, Winterbourn CC, Yin H, Halliwell B. Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. *Nat Metab.* 2022;4(6):651-662.

Schlattner U, Tokarska-Schlattner M, Wallimann T. Mitochondrial creatine kinase in human health and disease. *Biochim Biophys Acta.* 2006;1762(2):164-180.

Schröder K. NADPH oxidases in bone homeostasis and osteoporosis. *Free Radic Biol Med.* 2019;132:67-72.

Stemmler MP, Eccles RL, Brabletz S, Brabletz T. Non-redundant functions of EMT transcription factors. *Nat Cell Biol.* 2019;21(1):102-112.

Teitelbaum SL, Ross FP. Genetic regulation of osteoclast development and function. *Nat Rev Genet.* 2003;4(8):638-649.

Vandewalle C, Van Roy F, Berx G. The role of the ZEB family of transcription factors in development and disease. *Cell Mol Life Sci.* 2009;66(5):773-787.

Wallimann T, Tokarska-Schlattner M, Schlattner U. The creatine kinase system and pleiotropic effects of creatine. *Amino Acids.* 2011;40(5):1271-1296.

Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J.* 1992;281(Pt1)(Pt1):21-40.

Zhang K, Zhao H, Sheng Y, Chen X, Xu P, Wang J, Ji Z, He Y, Gao WQ, Zhu HH. Zeb1 sustains hematopoietic stem cell functions by suppressing mitofusin-2-mediated mitochondrial fusion. *Cell Death Dis.* 2022;13(8):735.

3rd Revision - Editorial Decision **2nd Feb 2023**

Dear Stephen,

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

Many thanks for trusting us with this manuscript; congratulations on publishing an exciting study!

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

You will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

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

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

** Click here to be directed to your login page: https://emboj.msubmit.net

EMBO Press Author Checklist

Corresponding Author Name: Stephen J. Weiss
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-111148R2

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Antibody catalogue numbers are specified in the "Materials and Methods" section.

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	siRNA and shRNA catalogue are specified in the "Materials and Methods" section.

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	The accession number of cell line are specified in the "Materials and Methods" section.
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	The detailed information of primary culture is specified in the "Materials and Methods" section.
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	The detailed information of Laboratory animals is provided in the "Materials and Methods" section. Zeb1f/f mice were provided by Thomas Brabletz (FAU University Erlangen-Nürnberg, Germany). Csf1r-Cre mice were obtained from Jackson Laboratories (stock nos. 0021024).
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Yes	The detailed information of animals is provided in the "Materials and Methods" section.
Please detail **housing and husbandry conditions**.	Yes	The housing and husbandry conditions is provided in the "Materials and Methods" section.

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	The service benefit from core facilities was mentioned in the "Acknowledgement" section.

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Yes	The detailed information is provided in the "Materials and Methods" section. All mouse work was performed under the guidelines and approval of the Animal Welfare and Ethics Committee (School and Hospital of Stomatology, Wuhan University) and the University of Michigan Institutional
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	The detailed sample size is provided in the "Figure legend" section. We used the sample sizes based upon previously reported data, preliminary and pilot data.
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	The detailed information is provided in the "Materials and Methods" section. For all animal experiments, mice were age-matched and then randomly assigned to minimize the effects of subjective bias.
Include a statement about **blinding** even if no blinding was done.	Yes	The statement about blinding is provided in the "Materials and Methods" section.
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	The statisical analysis performed was detailed in the "Materials and Methods" section.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	The replicate number of experiments is provided in the "Figure legend" section
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	The data replicates information is provided in the "Figure legend" section

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	All mouse work was performed under the guidelines and approval of the Animal Welfare and Ethics Committee (School and Hospital of Stomatology, Wuhan University) and the University of Michigan Institutional Animal Care & Use Committee (IACUC).
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	We have followed the ARRIVE guidelines through the course of these studies and during reporting.
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	The RNA sequencing data from this publication have been deposited to the Gene Expression Omnibus database at accession number GSE212302 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE212302).
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	The data citation of reused publicly available data is provided in the References section.