EMBO reports

Reducing oxidative protein folding alleviates senescence by minimizing ER-to-nucleus H2O2 release

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **9th Jan 2023**

Dear Prof. Wang

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

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns and have a number of suggestions on how to substantiate your conclusions that need to be addressed in the revision. I note that referee 2 suggests testing the physiological contribution of PDI using conditional mouse models. I agree that these analyses would strengthen your study but also note that the proposed experiments would involve a considerable time investment. I therefore suggest to include such data in case you have already started related experiments. Otherwise, this point can be addressed in the discussion.

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

We 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 (April 9th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

I am also happy to discuss the revision and proposed experiments further via e-mail or a video call, if you wish.

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

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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

When submitting your revised manuscript, we will require:

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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

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

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

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

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

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

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) At EMBO Press we ask authors to provide source data for the main figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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9) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

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 .

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

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The paper from Cheng et al provides evidence that the endoplasmic reticulum chaperone and oxidative protein folding factor PDI is involved in cellular senescence, and reveals some important mechanistic insight into the downstream drivers of the process.

For the most part the data are extensive, compelling and well controlled, but there are some experimental gaps and I am not completely convinced that the authors have always disentangled causative from correlative effects.

In Figure 1, the authors show that PDI is upregulated in late passage MSCs, Werner Syndrome MSCs, and aged mice livers but the full context and specificity to PDI is not clear. Are other members of the PDI family also upregulated? Is there a general upregulation of ER stress? In the PDI-/- ESCs (Fig 1D), it is also possible that premature stop codon transcripts, or the absence of PDI, could induce the unfolded protein response. Although some analysis of PDI family proteins has been done for the PDI-/- MSCs in Fig 2, I think it is important to blot for such indicator/control proteins in the Fig 1A-C experiments.

In Figure 2, the range of experimental approaches taken to confirm that senescence is delayed in PDI-/- MSCs appears convincing to me. However, in Figure 3, the use of the J chain to study oxidative protein folding is an unusual choice, as the MSCs would not normally produce immunoglobulins. Given that Werner Syndrome patients develop diabetes and atherosclerosis, insulin or LDLR folding might have been more informative.

The evidence that peroxide accumulates in the PDI-/- cells is solid, but I find it a jump to suggest that this same pool then causes the downstream effects seen in the nucleus. I am not convinced that the in vitro assay shown in 3H rules out the possibility that H_2O_2 from other sources plays a role in living cells. It is possible that mechanistically, there are different pathways at play in the PDI-/- ESCs and in Werner patient cells or the ageing liver.

The involvement of the p53 pathway is interesting (Figure 4 and page 8), as p53 has a strong zinc dependency; zinc is sensitive to redox states and has a key role in zinc-finger transcription factor activity; and the zinc-dependent chaperone ERp44 was upregulated in late passage MSC cells in Fig 1D. I wonder whether zinc status could be part of the link between PDI poise and the transcriptional changes seen in senescence - it would be valuable to know whether fluorescent reporters of zinc could shed some light on this possibility.

The identification and analysis of SERPINE1 in Fig 5 is exciting, but I would like to see a pulse-chase (or similar) assay to determine whether the oxidative protein folding and processing of SERPINE1 is directly altered in the PDI-/- MSCs.

Referee #2:

This interesting study focuses on the protein disulfide isomerase (PDI) and its contribution to human mesenchymal stem cells (hMSCs) aging. Authors demonstrated that deletion of PDI alleviated hMSCs senescence; knocking out PDI slowed the rate of oxidative protein folding and decreased the leakage of ER-derived H_2O_2 into the nucleus. Additionally, knocking out PDI attenuating SASP and SERPINE1 was identified as a key driver of cell senescence, regulated by the PDI-H_2O_2 axis.
All the experiments are well performed and controlled, and the data presented support the conclusions. There are minor points below that might be good to look at:

1. Authors demonstrate that PDI-knockout (PDI-/-) of hMSC alleviates senescence. To support this conclusion, several experiments have been done, and they demonstrated an increase in doubling levels, a decrease in SASP components, a decrease in DNA damage markers, and an increase in Ki67 levels, the marker of proliferation. MSCs have been implicated in the promotion of tumor growth in various cancer types. One important concern to consider is if PDI knockdown or inhibition in MSCs can contribute to tumorigenesis in the cell-autonomous or cell-non-autonomous manner.

2. What is the physiological contribution of these findings? Is it possible to target PDI in adult tissues of immunocompetent mice using conditional models?

3. Some of the abbreviations are not explained. Please add an abbreviation list.

Referee #3:

Fang Cheng and colleagues report that hydrogen peroxide generated in the endoplasmic reticulum during protein folding by protein disulfide isomerase (PDI) increases the rate by which human MSCs undergo senescence in vitro. They show that PDI knockout, while having no discernible ill-effect on cell health reduces peroxide production and in cells challenged with redox agent dithiothreitol, prevents leakage of peroxide into the nucleus. Subsequently, Fang Cheng and colleagues provide evidence that increased peroxide levels lead to SERPINE1 upregulation and that SERPINE1 overexpression drives cells into senescence. Their findings that peroxide generated during the physiological and constant process of protein folding induces senescence in human cells are significant in light of our poor understanding how senescence is induced in normal ageing. Moreover, the authors provide evidence that PDI is a more potent peroxide generation driver than other PDI-family members, while being functionally redundant with other PDI-family members. This provides broader insight into how differences in efficacy between enzymes performing similar cellular functions implicate cell health. Their findings are therefore of broader interest to the molecular biology community, but especially for those studying the fundamental processes driving senescence.

Fang Cheng and colleagues provide robust evidence for PDI being linked to an increased rate of cells undergoing senescence and for its role in ER peroxide production. They furthermore provide evidence that peroxide induces SERPINE1 and that SERPINE1 overexpression in turn induces senescence. What the authors could address more clearly is how these parts (PDI, SERPINE1 and senescence) are interlinked - after all, their stated aim at the start of the results is to "establish a link between cell senescence and PDI". I suggest that the authors rewrite their manuscript to clarify the order of events.
E.g., in Fig. 1 the authors report an increase in PDI in late passage MSC cultures (containing a high ratio of senescent cells) and old mice. However, it is not clear if the authors think this is cause (more PDI > more senescence) or consequence (more senescence> more PDI). Their latter data suggests PDI has a causal role in senescence, but this would then still leave the question open why it gets upregulated in late passage cultures/old mice to begin with. The links to SERPINE1 should also be better articulated: Does H_2O_2 generated by PDI enzymatic action cause nuclear DNA damage and thereby senescence, which then in turn induces SERPINE1? Or does H_2O_2 induce SERPINE1, which then induces senescence? The authors should speculate on how SERPINE1 expression is mechanistically linked to H_2O_2, why specifically nuclear H_2O_2 seems relevant here and finally how SERPINE1 induces senescence. While it would be out of the scope of this article to elucidate the relation of PDI, SERPINE1 and senescence with further experimental evidence, the authors should structure their arguments so that it is apparent how they think those parts are linked. Reworking the illustration in Fig. 6H to indicate the order of events would help.

The authors provide figures that are largely well designed, organised and labelled, showcasing an appropriate selection of data and where necessary using multiple methods to support their claims. Fang Cheng and colleagues should be commended for their extensive validation of their cell culture models and their concise description of peroxide generation during protein folding. Overall, I would recommend this manuscript for publication in EMBO reports but I would first ask the authors to please address the above mentioned restructuring of their arguments as well as the following points:

1. L42-44: The phrasing of the sentence implies a direct link between MSC functional decline and organ failure/ageing. There is no evidence for such link, partially because the function of adult MSCs in normal health is poorly understood. The authors should rephrase this sentence.

2. L202: Figure 3K-M and EV3G-K show that in presence of PDI H_2O_2 leaks into the cytoplasm and nucleus upon DTT treatment, while this does not occur in PDI-/- cells. In their concluding sentence the authors emphasize 'H_2O_2 generated by oxidative protein folding', however the PDI-/- cells do not differ in HyPer probe ratios in steady state (no DTT). This is surprising given that the authors show dramatic differences in H_2O_2 and ROS levels (Fig. 3E,F) between PDI wildtype and knockout cells. Could the authors please clarify why the HyPer probe does not indicate increased nuclear H_2O_2 at steady state, and if this is due to the limited sensitivity of the probe, provide data juxtaposing H_2O_2 measurements with the Amplex red assay and the HyPer probe to indicate what the sensitivity of the HyPer probe in their cell system is. This incongruence should also be referred to in the discussion (around L331), as the author's statement 'we clearly showed that the H_2O_2 generated by oxidative protein folding was released into the nucleus' is only warranted in DTT treated cells.

3. In Fig. 6D and F, the authors show that PDI knockout prevents senescence in cells treated with UV. This is surprising, since UV directly induces DNA damage and one would assume that reducing H_2O_2 release upon protein folding would not have a major impact on this. Could the authors please clarify how they think PDI knockout acts on UV-induced senescence? The authors should consider that an increase in proliferation rate with PDI knockout (as they show earlier) would compensate on population level for the UV-induced senescence induction.

4. Could the authors please clarify how PDI knockout would prevent telomere length loss in MSCs (especially at a 3x difference), which usually do not express TERT.

5. Could the authors please clarify how their results from the in vivo attrition assay are linked to MSC senescence?

6. Fig. 1 It would help if the authors already show the PDI expression data from Fig. 4K here.

7. The heatmaps in Fig. 4E and F are not useful, as no individual genes or gene groups are indicated. Instead heatmaps of a few relevant genes should been shown, with the gene names indicated. If the purpose was to show how the samples cluster (although the PCA already addresses this), in addition a heatmap with dendrogram could be shown.

8. The reversion of expression by PDI deletion the authors want to emphasize is not clear in the Venn diagrams from Fig. EV4J and I, please create a figure with a different plot format more suitable to showing reversion (e.g., line or point plots for selected genes) to accompany the Venn diagrams.

9. Fig. 4I and J: the symbols in the plots are indistinguishable and plot type makes it difficult to judge if the data supports the claims the authors make. Here a heatmap with one column per comparison (e.g., LP vs EP) might be more suitable.

10. Data on down- and up-regulated genes in primary human MSC replicative senescence has been published, with a list of regulated SASP genes (including SERPINE1) and key reference markers (https://doi.org/10.1007/s00018-021-04035-x; GSE152112). Cross-comparison of that dataset to the one of this study should shed light on how the changes induced by PDI knockout in ESC-derived MSCs relates to senescence in primary cells.

11. L248-250: The authors observe that SERPINE1 is upregulated in many tissues in older individuals and subsequently suggest it may be "a key driver of physiological aging". Since correlation does not imply causation and SERPINE1 may simply be one of the many genes that changes expression over age, I would suggest the authors avoid using the term driver here (which strongly suggests causality). Later on, when they show that SERPINE1 overexpression induces senescence, it is better justified in context of cellular ageing.

12. The authors state that their study "provides valuable cell models [for PDI knockout]". The authors should then clarify in their methods how other researchers can get access to the PDI-/- KO cells they generated and make their genetic constructs available (e.g., via Addgene).

13. In their discussion the authors speculate why a PDI knockout has no obvious negative effects on cell health, despite PDI being a key enzyme in protein folding. It would help if this point (redundancy) is alluded to already in the results, as I found it difficult to understand why differentiation of PDI-/- MSCs and ESCs is normal how the knockout of an enzyme essential for the folding of most membrane proteins (authors introduction) has no effect on expression of membrane proteins CD105, CD90 and CD73. Moreover, the authors should speculate in their discussion on why PDI knockout embryos are non-viable in light of the proposed redundancy and their data on PDI-/- ESC teratoma and in vitro differentiation.

14. In their methods, the authors state that chondrogenesis was assessed by 'Toluidine blue O', in the figure legends they state Alcian Blue. Please clarify which staining was used as the two dyes are not synonymous and give different staining patters.

15. Statements regarding ethical approval for use of primary human material (human gingiva tissues) missing.

16. Fig. EV2G: The authors should clarify how they assessed 'area'. The size of a cartilage pellet is not an appropriate indicator of chondrogenesis (size difference can be due to proliferation or chondrocyte hypertrophy), instead the Alcian Blue staining the authors performed (marking glycosaminoglycans) should be quantified.

17. L176: could the authors please provide a brief explanation of the role the -CGHC- active site has for PDI catalytic function. Since the +/-PDI oxygen consumption experiments (3I, 3J are embedded between cell culture experiments, it would help the reader interpret the data if the authors briefly mention in the results that this is a cell-free assay performed with purified proteins.

I spotted the following oversights, minor issues in style and spelling errors and figure displays (*delete*,):

1. Title: hydrogen peroxide preferred over H2O2 in the title
2. Abstract: By using the verb prevent instead of alleviate, the authors would put more emphasis on their key finding that ER-to-nucleus H2O2 release plays a role in inducing senescence.
3. L21: *was* accumulated
4. L24: SASP
5. L62: a byproduct
6. L64: for clarity: remove *a major process with high cellular energy consumption*
7. Fig. 4E: extracellular matrix misspelled (exrea)

8. L297: *pathological*, replace by more appropriate term
9. L318: *Although* (replace by yet or while after ,)
10. Fig. 2C Please clarify in legend: immunostaining for what? What is CRT?
11. Fig. 2J Please use a logarithmic scale for the mRNA values, as this makes it easier to parse the magnitude of gene downregulation (<1)
12. Fig. 3K/EV3G Using the one colour hue for PDI-/- and one colour hue for PDI+/+ lines (e.g., purple and cyan) with varying brightness per DTT dose would make parsing this plot easier.
13. Fig. 3M, EV3I,K Consider using a logarithmic scale for the ratios, as this makes it easier to interpret ratios below 1, and using a simple 2-or 3-colour gradient (e.g., red-to-blue or red-white-blue) instead of a rainbow scale, which is difficult to parse.
14. Fig. 4I,J It seems the bars in the y-axis label are indicating that these are absolute values, but this should be clarified in the legend. The distinction between down- and upregulated genes/proteins might be easier to assess by the plotting the -/+ log2 values.
15. Fig. EV2A: Please plot the FACS data using 2D histograms (with lines), instead of their '3D' format, as the perspective and overlapping solid shapes make assessing the shift of fluorescence intensity difficult.

Point-to-point response

We thank the three referees for their insightful comments and suggestions, which help us to significantly improve the manuscript. Below is a point-to-point response (blue text) embedded in the reviewers' comments.

Referee #1:

The paper from Cheng et al provides evidence that the endoplasmic reticulum chaperone and oxidative protein folding factor PDI is involved in cellular senescence, and reveals some important mechanistic insight into the downstream drivers of the process.

For the most part the data are extensive, compelling and well controlled, but there are some experimental gaps and I am not completely convinced that the authors have always disentangled causative from correlative effects.

We thank this reviewer for the overall positive notes.

1. In Figure 1, the authors show that PDI is upregulated in late passage MSCs, Werner Syndrome MSCs, and aged mice livers but the full context and specificity to PDI is not clear. Are other members of the PDI family also upregulated? Is there a general upregulation of ER stress? In the $PDI^{-/-}$ ESCs (Fig 1D), it is also possible that premature stop codon transcripts, or the absence of PDI, could induce the unfolded protein response. Although some analysis of PDI family proteins has been done for the $PDI^{-/-}$ MSCs in Fig 2, I think it is important to blot for such indicator/control proteins in the Fig 1A-C experiments.

Response:

We thank the reviewer for these insightful suggestions. We have detected the protein levels of PDI family proteins including P5, ERp46, ERp72, ERp57 and ERp46 in the replicative senescent, Werner Syndrome (WS) hMSCs and the livers of mice. Results showed that only ERp57 and ERp44 were upregulated in both hMSCs aging models (Response Figure 1A-D). However, neither of PDI homologs was upregulated in the livers of aged mice (Response Figure 1E-F). Besides, we observed that the expression of ER stress marker BiP was upregulated in the WS hMSCs but not in the replicative senescent hMSCs and the livers of aged mice (Response Figure 1A-F). In addition, we evaluated the secretion levels of PDI and PDI family members in various SASPs from multiple senescence inducers and in plasma proteome from 3,087 healthy individuals (Response Figure 1I). The results showed that only the secretion of PDI was increased in all different contexts. Thus, the upregulation of PDI in different aging models is conserved (Figure 1), highlighting the critical role of PDI in the regulation of aging. Due to space limitations, we choose to include Response Figure 1I in the revised Figure EV1.

Our unpublished data showed that PDI deficiency induced the activation of the IRE1 branch in the hESCs and hMSCs. Specifically, the depletion of PDI elevated the phosphorylation of

IRE1α and its downstream substrate spliced XBP-1 (XBP-1s), as well as the phosphorylation of PREK substrate eIF2α (Response Figure 1G and 1H). Depletion of PDI resulted in the upregulation of the most abundant ER chaperone, BiP (Response Figure 1G and 1H) [Figures for referees not shown.], which may contribute to the restoration of ER homeostasis in the absence of PDI. The activation of the adaptive UPR could be another reason for the beneficial effect of PDI depletion. A previous study showed that IRE1α-XBP-1 pathway activation declines during aging and that exogenous activation of XBP-1 increases the longevity of nematodes (Taylor & Dillin, 2013). The possible role of UPR in $PDI^{-/-}$ hMSC is for sure very important and will be further studied in our lab.

2. In Figure 2, the range of experimental approaches taken to confirm that senescence is delayed in PDI$^{-/-}$ MSCs appears convincing to me. However, in Figure 3, the use of the J chain to study oxidative protein folding is an unusual choice, as the MSCs would not normally produce immunoglobulins. Given that Werner Syndrome patients develop diabetes and atherosclerosis, insulin or LDLR folding might have been more informative.

Response:

We thank the reviewer for this constructive suggestion. Immunoglobulin J-chain is used as a model substrate of oxidative protein folding for a long time (Mezghrani *et al*, 2001). According to the reviewer's comment, we also detect the oxidative folding of LDLR. Because the full-length LDLR is too large, we constructed a truncated form of LDLR (LDLR-236) including 5 cysteine-rich domains (R1-R5), each stabilized by three disulfide bonds (Response Figure 2A) (Kadokura *et al*, 2020). As expected, deficiency of PDI decreased the rate at which the reduced

LDLR-236 disappeared (Response Figure 2B)[Figures for referees not shown.]. Besides, we also detected the oxidative folding of roGFP-ER, which contains one disulfide bond (Response Figure 2C)[Figures for referees not shown.]. The results showed that deficiency of PDI also slowed the rate of roGFP-ER oxidative folding (Response Figure 2D). The data about LDLR has been included in the revised Figure EV3. We added in the text –" Similar to the folding of J-chain, loss of PDI also slowed the oxidative folding rate of low-density lipoprotein receptor (LDLR), a cysteine-rich protein (Figure EV3E) (Kadokura *et al.*, 2020)." (Page 6, Line 162-164)

(C) The reduced and oxidized form of roGFP-ER.

(D) Oxidative folding of roGFP-ER. $PDI^{+/+}$ and $PDI^{-/-}$ hMSCs were transfected with roGFP-ER for 48 hr, then were pulsed with 5 mM DTT and chased at indicated time after DTT removal.

The lysates were analyzed using non-reducing SDS-10% PAGE and α-FLAG western blotting.

The mobility of reduced roGFP-ER monomers (Red), oxidized monomers (Oxi) are indicated.

Right, the fraction of reduced roGFP-ER was quantified by densitometry. Data are shown as

mean ±SEM, *n* = 3 independent experiments, two-tailed Student's t-test.

3. The evidence that peroxide accumulates in the $PDI^{-/-}$ cells is solid, but I find it a jump to

suggest that this same pool then causes the downstream effects seen in the nucleus. I am not

convinced that the in vitro assay shown in 3H rules out the possibility that H_2O_2 from other sources plays a role in living cells. It is possible that mechanistically, there are different

pathways at play in the $PDI^{-/-}$ ESCs and in Werner patient cells or the ageing liver. **Response:** We thank the reviewer for this comment. We agree with the reviewer that there are different H_2O_2 sources in living cells. Oxidases such as Ero1, QSOX, and NOX4 are the possible sources of H_2O_2 at the ER, of which Ero1-PDI mediated oxidative protein folding is recognized as the main source of H_2O_2 (Roscoe & Sevier, 2020). Data in Figure 3K-M showed that the HyPer7 probe was not oxidized at all in $PDI^{-/-}$ hMSCs upon DTT challenge, indicating that the ER-to-nucleus release of H_2O_2 was mediated by PDI. Since PDI cannot be oxidized by QSOX or NOX4 (Bulleid & Ellgaard, 2011), we believe that Ero1-PDI produced H_2O_2 is a main source in our system. A reconstituted system shown in Figure 3H further emphasized the importance of PDI, rather than its homologs, in the production of H_2O_2. Moreover, we observed that the knockdown of Ero1α in WT hMSCs also decreased H_2O_2 release from the ER to the cytosol or nucleus (Response Figure 3A-C)[Figures for referees not shown.]. Altogether, our results indicated that Ero1α-PDI mediated oxidative protein folding is a major source for ER-to-nucleus H_2O_2 release.

4. The involvement of the p53 pathway is interesting (Figure 4 and page 8), as p53 has a strong zinc dependency; zinc is sensitive to redox states and has a key role in zinc-finger transcription factor activity; and the zinc-dependent chaperone ERp44 was upregulated in late passage MSC cells in Fig 2D. I wonder whether zinc status could be part of the link between PDI poise and

the transcriptional changes seen in senescence - it would be valuable to know whether6

fluorescent reporters of zinc could shed some light on this possibility.

Response:

We thank the reviewer for raising this insightful point. We have detected the levels of zinc in $PDI^{+/+}$ and $PDI^{-/-}$ hMSCs at early and late passage using the probe Zinyr-1. The results showed that the zinc levels were increased at late passage hMSCs. Additionally, decreased zinc levels were detected in $PDI^{-/-}$ hMSCs (Response Figure 4)[Figures for referees not shown.]. Thus, the upregulated of ERp44 may be related to the increased levels of zinc. The role of zinc in ER homeostasis and aging is a very interesting question, which needs to be investigated in the future.

5. The identification and analysis of SERPINE1 in Fig 5 is exciting, but I would like to see a pulse-chase (or similar) assay to determine whether the oxidative protein folding and processing of SERPINE1 is directly altered in the $PDI^{-/-}$ MSCs.

Response:

We thank the reviewer for this suggestion. There is no cysteine residue in the mature SERPINE1, except one in the signal sequence. According to the reviewer's suggestion, we did a pulse-chase assay to detect the folding process of SERPINE1, and we did not observe any intra- or inter-molecular disulfide. Again, endogenous SERPINE1 was largely downregulated in $PDI^{-/-}$ hMSCs (Response Figure 5)[Figures for referees not shown.].

Referee #2:

This interesting study focuses on the protein disulfide isomerase (PDI) and its contribution to human mesenchymal stem cells (hMSCs) aging. Authors demonstrated that deletion of PDI alleviated hMSCs senescence; knocking out PDI slowed the rate of oxidative protein folding and decreased the leakage of ER-derived H_2O_2 into the nucleus. Additionally, knocking out PDI attenuating SASP and SERPINE1 was identified as a key driver of cell senescence, regulated by the PDI-H_2O_2 axis.

All the experiments are well performed and controlled, and the data presented support the conclusions. There are minor points below that might be good to look at:

We thank this reviewer for these positive notes.

1. Authors demonstrate that PDI-knockout ($PDI^{-/-}$) of hMSC alleviates senescence. To support

this conclusion, several experiments have been done, and they demonstrated an increase in doubling levels, a decrease in SASP components, a decrease in DNA damage markers, and an increase in Ki67 levels, the marker of proliferation. MSCs have been implicated in the promotion of tumor growth in various cancer types. One important concern to consider is if PDI knockdown or inhibition in MSCs can contribute to tumorigenesis in the cell-autonomous or cell-non-autonomous manner.

Response:

We understand the reviewer's concerns and thank for the insightful question. Tumorigenesis is the gain of malignant properties in normal cells, including fast proliferation, metastasis and genomic instability, etc., which has been recognized as the hallmarks of cancer (Cao, 2017).

Our result in Figure 2E showed that although deficiency of PDI increased the doubling levels, there was a growth arrest at passage 12 in $PDI^{-/-}$ hMSCs. Besides, data in Figure 2N indicated that $PDI^{-/-}$ hMSCs exhibited delayed *in vivo* attrition, but still decayed at day 7 (Response Figure 6A), suggesting that deficiency of PDI did not cause tumor formation *in vivo*. Besides, CNV analysis in Figure EV2C showed that $PDI^{-/-}$ hMSCs maintained genomic integrity. Meantime, we checked the expression levels of tumor-related genes upon PDI deletion by RNA-Seq and observed a minimal global change in the expression of tumor-related genes (from KEGG, hsa05202) (Response Figure 6B). Furthermore, to check whether PDI inhibition in hMSCs can contribute to tumorigenesis in a cell-non-autonomous manner, we detected the effect of conditional medium of $PDI^{+/+}$ and $PDI^{-/-}$ hMSCs on the migration and invasion of cervical cancer HeLa cells. The results showed that deficiency of PDI did not promote the migration or invasion of HeLa cells (Response Figure 6C and 6D)[Figures for referees not shown.].

2. What is the physiological contribution of these findings? Is it possible to target PDI in adult tissues of immunocompetent mice using conditional models?

Response:

We thank the reviewer for this point. PDI-knockout mice are embryonic lethal. We agree that it would be interesting and possible to test the physiological contribution of PDI using conditional mouse models. However, these animal experiments would involve a considerable time and funding investment. Instead, we have included more discussion about the physiological meaning of our findings in the revised manuscript.

"Thus, our study provides valuable cell models for exploring the biological function of PDI in various human stem cells and a potential strategy for generating genetic enhancement human stem cells for cell therapies." (Page 12 Line 349-351).

"Recently, PDI has emerged as a potential target for cancer and thrombosis treatment (Bekendam & Flaumenhaft, 2016; Wang et al, 2021; Xu et al, 2014). It will be interesting to investigate whether anticancer and antithrombotic PDI inhibitors can be used for aging intervention." (Page 12 Line 354-357).

"Moreover, as both PDI (Kim *et al*, 2013; Wang *et al*, 2022) and SERPINE1 (Hisada *et al*, 2021; Westrick & Eitzman, 2007) have been well documented to be associated with thrombosis, targeting the PDI-H_2O_2-SERPINE1 axis may be a more effective strategy for the prevention and treatment of vascular disease." (Page 13 368-371)

3. Some of the abbreviations are not explained. Please add an abbreviation list.

Response:

We thank the reviewer for this suggestion. We have checked all the abbreviations and made explanations. An abbreviation list was added in the revised manuscript.

Referee #3:

Fang Cheng and colleagues report that hydrogen peroxide generated in the endoplasmic reticulum during protein folding by protein disulfide isomerase (PDI) increases the rate by which human MSCs undergo senescence in vitro. They show that PDI knockout, while having no discernible ill-effect on cell health reduces peroxide production and in cells challenged with redox agent dithiothreitol, prevents leakage of peroxide into the nucleus. Subsequently, Fang Cheng and colleagues provide evidence that increased peroxide levels lead to SERPINE1 upregulation and that SERPINE1 overexpression drives cells into senescence.

Their findings that peroxide generated during the physiological and constant process of protein folding induces senescence in human cells are significant in light of our poor understanding how senescence is induced in normal ageing. Moreover, the authors provide evidence that PDI

is a more potent peroxide generation driver than other PDI-family members, while being functionally redundant with other PDI-family members. This provides broader insight into how differences in efficacy between enzymes performing similar cellular functions implicate cell health. Their findings are therefore of broader interest to the molecular biology community, but especially for those studying the fundamental processes driving senescence.

We thank the reviewer for these positive comments.

Fang Cheng and colleagues provide robust evidence for PDI being linked to an increased rate of cells undergoing senescence and for its role in ER peroxide production. They furthermore provide evidence that peroxide induces SERPINE1 and that SERPINE1 overexpression in turn induces senescence. What the authors could address more clearly is how these parts (PDI, SERPINE1 and senescence) are interlinked - after all, their stated aim at the start of the results is to "establish a link between cell senescence and PDI". I suggest that the authors rewrite their manuscript to clarify the order of events.

E.g., in Fig. 1 the authors report an increase in PDI in late passage MSC cultures (containing a high ratio of senescent cells) and old mice. However, it is not clear if the authors think this is cause (more PDI > more senescence) or consequence (more senescence> more PDI). Their latter data suggests PDI has a causal role in senescence, but this would then still leave the question open why it gets upregulated in late passage cultures/old mice to begin with. The links to SERPINE1 should also be better articulated: Does H_2O_2 generated by PDI enzymatic action cause nuclear DNA damage and thereby senescence, which then in turn induces SERPINE1? Or does H_2O_2 induce SERPINE1, which then induces senescence? The authors should speculate on how SERPINE1 expression is mechanistically linked to H_2O_2, why specifically nuclear H_2O_2 seems relevant here and finally how SERPINE1 induces senescence. While it would be out of the scope of this article to elucidate the relation of PDI, SERPINE1 and senescence with further experimental evidence, the authors should structure their arguments so that it is apparent how they think those parts are linked. Reworking the illustration in Fig. 6H to indicate the order of events would help.

The authors provide figures that are largely well designed, organised and labelled, showcasing an appropriate selection of data and where necessary using multiple methods to support their claims. Fang Cheng and colleagues should be commended for their extensive validation of their cell culture models and their concise description of peroxide generation during protein folding.

Overall, I would recommend this manuscript for publication in EMBO reports but I would first ask the authors to please address the above mentioned restructuring of their arguments as well as the following points:

Response:

We thank the reviewer for these positive and constructive comments. The data in Figure 1 showed that expression of PDI was upregulated in replicative, premature senescent hMSCs and livers of old mice, suggesting that there was a link between PDI and senescence. Further, our data confirmed that PDI knockout/knockdown in various cell aging models alleviates hMSCs senescence, indicating that PDI plays a causal role in senescence.

As the reviewer pointed out, why the expression of PDI (and many other proteins) is altered during the aging process is an open question. On the one hand, the accumulation of misfolded proteins in the aging process leads to the imbalance of ER protein homeostasis, which could induce the expression of PDI, possibly through adaptive UPR signaling. The excessive activation of Ero1-PDI system causes the accumulation of byproduct H_2O_2, further augments the oxidative damage to aged cells. On the other hand, it may be subject to epigenetic regulation. A recent study showed that faithful double-stranded DNA break repair erodes the epigenetic landscape and accelerates the hallmarks of aging (Yang *et al*, 2023). Epigenetic changes during the aging process could also lead to the upregulation of PDI expression. Again, increased H_2O_2 release by Ero1-PDI could further augment DNA damage and accelerate aging.

Aging is a complex phenotype, there is still much debate about which hallmarks are primary drivers, secondary mediators, or simply downstream consequences of the aging process (Teefy & Benayoun, 2023). According to our and others' research, SERPINE1 expression is regulated via H_2O_2 (Oszajca et al, 2008; Swiatkowska et al, 2002). Moreover, CRISPR activation of endogenous SERPINE1 expression in $PDI^{-/-}$ hMSCs accelerated cell senescence (Fig 5C-G), suggesting that SERPINE1 acts downstream of PDI-H_2O_2 axis to mediate cell aging. Previous studies have shown that oxidative stress-related transcription factors such as NF-κB, P53, and HIF-1 in the nucleus regulate the expression of SERPINE1 (Oszajca *et al.*, 2008; Rahman & Krause, 2020). However, we were unable to detect binding between NF-κB and SERPINE1 promoter using ChIP-qPCR (data not shown), indicating that there may be other oxidative stress-related transcription factors that regulate the expression of SERPINE1. Previous research has shown that SERPINE1 governs cellular senescence by regulating the extracellular proteolysis of SASP. Elzi and colleagues identified that the SERPINE1-IGFBP3 signaling pathway induces cell senescence of mesenchymal stromal cells (Elzi *et al*, 2012). In our study, we found that the expression of IGFBP3 was downregulated in $PDI^{-/-}$ hMSCs (data not shown). Thus, SERPINE1 may induce cell senescence via downregulating the expression of IGFBP3. Although there are still many interesting questions to be answered with further experimental evidence, we believe our findings that the PDI-H_2O_2-SERPINE1 axis plays a key role in cell senescence will bring new insights into the link between oxidative protein folding and aging.

We have rewritten our manuscript according to the statements above mentioned in the revised manuscript (Line 26-27, 78-79, 303-304). We have also reworked the illustration in Fig. 6H to indicate the order of events (PDI-->H_2O_2-->SERPINE1-->senescence).

1. L42-44: The phrasing of the sentence implies a direct link between MSC functional decline and organ failure/ageing. There is no evidence for such link, partially because the function of adult MSCs in normal health is poorly understood. The authors should rephrase this sentence.

Response:

We have rephrased this sentence in the revised manuscript "However, the functions of MSCs progressively decline during aging, a process involving lost maintenance of tissue homeostasis, which induces aging-associated tissue degeneration." (Page 2 Line 43-45).

2. L202: Figure 3K-M and EV3G-K show that in presence of PDI H_2O_2 leaks into the cytoplasm and nucleus upon DTT treatment, while this does not occur in $PDI^{-/-}$ cells. In their concluding sentence the authors emphasize 'H_2O_2 generated by oxidative protein folding', however the $PDI^{-/-}$ cells do not differ in HyPer probe ratios in steady state (no DTT). This is surprising given that the authors show dramatic differences in H_2O_2 and ROS levels (Fig. 3E and F) between PDI wildtype and knockout cells. Could the authors please clarify why the HyPer probe does not indicate increased nuclear H_2O_2 at steady state, and if this is due to the limited sensitivity of the probe, provide data juxtaposing H_2O_2 measurements with the Amplex red assay and the HyPer probe to indicate what the sensitivity of the HyPer probe in their cell system is. This incongruence should also be referred to in the discussion (around L331), as the author's statement 'we clearly showed that the H_2O_2 generated by oxidative protein folding was released into the nucleus' is only warranted in DTT treated cells.

Response:

We thank the reviewer for this comment. In this study, both Amplex Red and HyPer7 were used to detect the H_2O_2 levels in cells. Amplex Red reagent is a colorless substrate that reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin (excitation/emission maxima=570/585 nm) by HRP. Amplex Red reaction can be used to routinely detect nanomolar H_2O_2 levels, which is very sensitive (Amplex Red Enzyme Assays, Invitrogen). In this study, Amplex Red was used to detect the total cellular H_2O_2 levels after cells being harvested and lysed (see Methods). We have also rewritten the sentence-- "An Amplex Red assay confirmed the decreased overall H_2O_2 levels in the lysates of $PDI^{-/-}$ hMSCs (Fig 3E)." (Page 6 Line 175-176)

Different from Amplex Red, HyPer7 was used to detect H_2O_2 levels in the subcellular organelle in living cells. HyPer7 is a H_2O_2 probe based on cpYFP integrated into the OxyR-RD domain from *Neisseria meningitides* (Pak *et al*, 2020). HyPer7 can be used to detect micromolar H_2O_2 levels (Pak *et al.*, 2020), which is less sensitive compared to Amplex Red. Besides, as the cytosol and nucleus are in a reducing environment and composed of many peroxidases and

peroxiredoxins, the HyPer7 probes in the cytosol or nucleus were mainly reduced at resting state. Nevertheless, compared with Amplex Red, HyPer7 was suitable to detect the release of H_2O_2 from the ER to nucleus under reductive stress (e.g. DTT challenge) in living cells. To our knowledge, this is the first report showing that there is an ER-to-nucleus release of H_2O_2 in cells with a validated probe.

3. In Fig. 6D and F, the authors show that PDI knockout prevents senescence in cells treated with UV. This is surprising, since UV directly induces DNA damage and one would assume that reducing H_2O_2 release upon protein folding would not have a major impact on this. Could the authors please clarify how they think PDI knockout acts on UV-induced senescence? The authors should consider that an increase in proliferation rate with PDI knockout (as they show earlier) would compensate on population level for the UV-induced senescence induction.

Response:

Agree. $PDI^{-/-}$ hMSCs may be more resistant to senescence-induced stress, including UV treatment, probably due to an increase in proliferation rate. We have added in the legend of the revised manuscript "(D, F) Single clonal formation assay (D) and SA-β-gal staining assay (F) in RS hMSCs transduced with sgNTC or *PDI*-targeting sgRNA after treatment with UV irradiation (10 J/m^2) and then cultured for another 2 days. n = 3 biological repeats." (Page 45, Line 1155-1157)

4. Could the authors please clarify how PDI knockout would prevent telomere length loss in MSCs (especially at a 3x difference), which usually do not express TERT.

Response:

We thank the reviewer for this comment. Telomere attrition is one of the hallmarks of aging and telomeres shorten with age (Lopez-Otin *et al*, 2023). Besides, the rate of telomere shortening indicates the pace of aging (Shammas, 2011). Our results showed that the rate of telomere shortening in $PDI^{-/-}$ hMSCs was slower than that in $PDI^{+/+}$ hMSCs, not getting longer. To avoid misunderstanding, the telomere length was normalized to $PDI^{-/-}$ hMSCs in the revised Figure 2L.

5. Could the authors please clarify how their results from the in vivo attrition assay are linked to MSC senescence?

Response:

According to the published papers, the tibialis anterior muscle of mice has a microenvironment suitable for the survival of mesoderm cells and there is an accelerated attrition of senescent MSCs *in vivo* (Deng et al, 2019; Pan et al, 2016). Thus, the rate of cell attrition *in vivo* indicates the pace of MSCs senescence.

6. Fig. 1 It would help if the authors already show the PDI expression data from Fig. 4K here.

Response:

As suggested, we evaluated the secretion levels of PDI and PDI family members in various SASPs from multiple senescence inducers and in plasma proteome from 3,087 healthy individuals in Figure EV1A. We found the secretion levels of PDI were all increased in different contexts. Please also see our response to Reviewer #1, point 1.

7. The heatmaps in Fig. 4E and F are not useful, as no individual genes or gene groups are indicated. Instead heatmaps of a few relevant genes should been shown, with the gene names indicated. If the purpose was to show how the samples cluster (although the PCA already addresses this), in addition a heatmap with dendrogram could be shown.

Response:

We thank the reviewer for the kind reminders. In Fig. 4E and F, the purpose was to show the expression and enriched pathways of rescued genes in PDI knockout hMSCs. As suggested, we also added some examples of rescued genes in enriched pathways in the revised Figure 4E and 4F.

8. The reversion of expression by PDI deletion the authors want to emphasize is not clear in the Venn diagrams from Fig. EV4J and I, please create a figure with a different plot format more suitable to showing reversion (e.g., line or point plots for selected genes) to accompany the Venn diagrams.

Response:

As suggested, we have added the heatmaps in new Figures 4I and 4J, showing the genes with reversed expression after PDI deletion at both the proteomic and transcriptional levels, and highlighting the aging-related genes (red color).

9. Fig. 4I and J: the symbols in the plots are indistinguishable and plot type makes it difficult to judge if the data supports the claims the authors make. Here a heatmap with one column per comparison (e.g., LP vs EP) might be more suitable.

Response:

Done. Please see above.

10. Data on down- and up-regulated genes in primary human MSC replicative senescence has been published, with a list of regulated SASP genes (including SERPINE1) and key reference markers (https://doi.org/10.1007/s00018-021-04035-x; GSE152112). Cross-comparison of that

dataset to the one of this study should shed light on how the changes induced by PDI knockout in ESC-derived MSCs relates to senescence in primary cells.

Response:

We thank the reviewer for the insightful comments. Through cross-comparison of age-related DEGs (P4 vs. P1) in GSE152112 of that study, we found that PDI deficiency partially restored the expression of age-related DEGs (Response Figure 7A and B). A biology pathway analysis showed that DEGs enriched in “Angiogenesis”, “Response to oxygen levels”, and “Endothelia cell migration” were upregulated during cell senescence and reversed by PDI depletion (Response Figure 7C). In contrast, DEGs enriched in “Cell cycle” and “DNA repair” that had been downregulated during cell senescence were reactivated by PDI deletion (Response Figure 7D)[Figures for referees not shown.].

11. L248-250: The authors observe that SERPINE1 is upregulated in many tissues in older individuals and subsequently suggest it may be "a key driver of physiological aging". Since correlation does not imply causation and SERPINE1 may simply be one of the many genes that changes expression over age, I would suggest the authors avoid using the term driver here

(which strongly suggests causality). Later on, when they show that SERPINE1 overexpression

induces senescence, it is better justified in context of cellular ageing.

Response:

We thank the reviewer for this suggestion. We have replaced the sentence "*SERPINE1* may be a key driver of physiological aging" with "*SERPINE1* is closely related with physiological aging" in the revised manuscript. (Page 9 Line 254-255) In addition, when we show that SERPINE1 overexpression induces senescence later on, we carefully justified in context of cellular senescence.

12. The authors state that their study "provides valuable cell models [for PDI knockout]". The authors should then clarify in their methods how other researchers can get access to the $PDI^{-/-}$ KO cells they generated and make their genetic constructs available (e.g., via Addgene).

Response:

We have written in the 'Data availability' part in the Methods -- "Other data or materials generated in this study are available from the corresponding authors upon reasonable request". (Page 25, Line 740-741)

13. In their discussion the authors speculate why a PDI knockout has no obvious negative effects on cell health, despite PDI being a key enzyme in protein folding. It would help if this point (redundancy) is alluded to already in the results, as I found it difficult to understand why differentiation of $PDI^{-/-}$ MSCs and ESCs is normal how the knockout of an enzyme essential for the folding of most membrane proteins (authors introduction) has no effect on expression of membrane proteins CD105, CD90 and CD73. Moreover, the authors should speculate in their discussion on why PDI knockout embryos are non-viable in light of the proposed redundancy and their data on PDI-/- ESC teratoma and in vitro differentiation.

Response:

We thank the reviewer for the helpful suggestion. We have added the point of redundancy in the results section of the revised manuscript -- "The redundancy of PDI family proteins may account for the acquisition of PDI-deficient hMSCs." (Page 5, Line 122-123)

PDI knock-out mice have lethal phenotypes occurring at E9.5 (https://www.mousephenotype.org/data/genes/MGI:97464), but hESCs are derived from the inner cell mass of blastocyst-stage embryos, which is earlier than E9.5. Thus, we can obtain PDI knockout hESCs successfully. PDI-deficient hESCs could form the teratoma, indicating that these hESCs maintained pluripotency. However, this pluripotency does not guarantee the normal development of various organs *in vivo* or *in vitro*. PDI knockout embryos are non-viable, indicating that PDI plays an important role in a certain stage of embryonic development that cannot be compensated by redundancy. According to our unpublished data, $PDI^{-/-}$ hESCs cannot

be differentiated into human vascular endothelial cells (hVECs) efficiently, while PDI$^{-/-}$ human neural stem cells (hNSCs) and hMSCs were available, indicating that PDI may play an important role in vascular formation. We have added this point to the discussion -- "However, this outcome does not mean that PDI is dispensable in all developmental stage and all types of cells. For example, the PDI knock-out mice have lethal phenotypes occurring at E9.5 (https://www.mousephenotype.org/data/genes/MGI:97464), which is later than the blastocyst-stage where the hESCs derived, and PDI$^{-/-}$ hESCs cannot be differentiated into human vascular endothelial cell (hVECs) efficiently (our unpublished observation), suggesting that PDI may play an important role in angiogenesis." (Page 11 Line 316-321)

14. In their methods, the authors state that chondrogenesis was assessed by 'Toluidine blue O', in the figure legends they state Alcian Blue. Please clarify which staining was used as the two dyes are not synonymous and give different staining patters.

Response:

We are sorry for this mistake. In this study the chondrogenesis was assessed by Toluidine blue staining. We have corrected in the legend of Figure EV2G.

15. Statements regarding ethical approval for use of primary human material (human gingiva tissues) missing.

Response:

We thank the reviewer for this reminder. We have added the ethical approval for use of primary human material (human gingiva tissues) in the methods section of our revised manuscript. (Page 15-16 Line 431-433)

16. Fig. EV2G: The authors should clarify how they assessed 'area'. The size of a cartilage pellet is not an appropriate indicator of chondrogenesis (size difference can be due to proliferation or chondrocyte hypertrophy), instead the Alcian Blue staining the authors performed (marking glycosaminoglycans) should be quantified.

Response:

In the original manuscript, we assessed the size of cartilage pellet by measuring the diameter. According to the reviewer's suggestion, we have quantified the intensity of Toluidine blue staining in Figure EV2G.

17. L176: could the authors please provide a brief explanation of the role the -CGHC- active site has for PDI catalytic function. Since the +/-PDI oxygen consumption experiments (3I, 3J are embedded between cell culture experiments, it would help the reader interpret the data if

the authors briefly mention in the results that this is a cell-free assay performed with purified proteins.

Response:

We thank the reviewer for this helpful suggestion. We have explained the role of the -CGHC- active site in the revised manuscript -- "In addition to PDI, four other constitutively expressed PDI family members, P5, ERp46, ERp72, and ERp57, each of which harbors at least two -CGHC- active sites critical for catalyzing the thiol-disulfide interchange reaction". (Page 7 Line 181-183) We also clarified – "we reconstituted the oxidative protein folding system *in vitro* with purified proteins." (Page 6 Line 180)

I spotted the following oversights, minor issues in style and spelling errors and figure displays (*delete*,):

1. Title: hydrogen peroxide preferred over H_2O_2 in the title

Response:

Sorry, replacement of 'H_2O_2' with 'hydrogen peroxide' will exceed the character limit.

2. Abstract: By using the verb prevent instead of alleviate, the authors would put more emphasis on their key finding that ER-to-nucleus H_2O_2 release plays a role in inducing senescence.

Response:

We thank the reviewer for this kind comment. Considering that PDI knockout slows but not stops aging, we think the verb "alleviate" may be more suitable.

3. L21: *was* accumulated

Response:

Corrected.

4. L24: SASP

Response:

We have rephrased this sentence in the abstract.

5. L62: a byproduct

Response:

Corrected.

6. L64: for clarity: remove *a major process with high cellular energy consumption*

Response:

Corrected.

7. Fig. 4E: extracellular matrix misspelled (exrea)

Response:

Corrected.

8. L297: *pathological*, replace by more appropriate term

Response:

We have replaced the word “pathological” with “premature” in the revised manuscript.

9. L318: *Although* (replace by yet or while after ,)

Response:

We have replaced the word “Although” with “Yet” in the revised manuscript.

10. Fig. 2C Please clarify in legend: immunostaining for what? What is CRT?

Response:

We have revised the legend of Figure 2C - “Immunostaining of PDI and calreticulin (CRT) in $PDI^{+/+}$ and $PDI^{-/-}$ hMSCs, CRT was used as ER marker. Scale bar, 25 μm.” (Page 34 Line 991-992)

11. Fig. 2J Please use a logarithmic scale for the mRNA values, as this makes it easier to parse the magnitude of gene downregulation (<1)

Response:

According to the reviewer’s suggestion, we used a logarithmic scale for the mRNA values in revised Figure 2J.

12. Fig. 3K/EV3G Using the one colour hue for $PDI^{-/-}$ and one colour hue for $PDI^{+/+}$ lines (e.g., purple and cyan) with varying brightness per DTT dose would make parsing this plot easier.

Response:

We thank the reviewer for the kind suggestion. We used the purple hue for $PDI^{+/+}$ and cyan hue for $PDI^{-/-}$ in the revised Figure 3K and Figure EV3H.

13. Fig. 3M, EV3I, K Consider using a logarithmic scale for the ratios, as this makes it easier to interpret ratios below 1, and using a simple 2-or 3-colour gradient (e.g., red-to-blue or red-white-blue) instead of a rainbow scale, which is difficult to parse.

Response:

We thank the reviewer for this suggestion. If the purpose was to show the fold change of H_2O_2

levels that between $PDI^{-/-}$ and $PDI^{+/+}$ hMSCs, a logarithmic scale for the ratios is more suitable. While the ratios in Fig. 3M, EV3I, K reflect the fluorescence intensity at 488/405 nm excitation, which indicating the H_2O_2 levels in living cells. Thus, displaying the original values of ratio may be more appropriate, and can be used for comparison with other published works. According to our results and others (Pak *et al.*, 2020), there is a gradient of H_2O_2 levels in living cells, so the rainbow scale can better reflect this gradient change.

14. Fig. 4I, J It seems the bars in the y-axis label are indicating that these are absolute values, but this should be clarified in the legend. The distinction between down- and upregulated genes/proteins might be easier to assess by the plotting the -/+ log2 values.

Response:

Figure 4I and J have been replaced with heatmaps in the revised figure. The distinction between down- and upregulated genes/proteins is now clearly indicated by plotting the Log_2(Fold Change) values.

15. Fig. EV2A: Please plot the FACS data using 2D histograms (with lines), instead of their '3D' format, as the perspective and overlapping solid shapes make assessing the shift of fluorescence intensity difficult.

Response:

According to the reviewer's suggestion, we have shown the FACS data using 2D histograms in the revised Figure EV2A and B.

Again, we sincerely thank Referee #3 for careful reading of our manuscript.

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Wang L, Yu J, Wang CC (2021) Protein disulfide isomerase is regulated in multiple ways: Consequences for conformation, activities, and pathophysiological functions. *Bioessays* 43: e2000147

Westrick RJ, Eitzman DT (2007) Plasminogen activator inhibitor-1 in vascular thrombosis. *Curr Drug Targets* 8: 966-1002

Xu S, Sankar S, Neamati N (2014) Protein disulfide isomerase: a promising target for cancer therapy. *Drug Discov Today* 19: 222-240

Yang JH, Hayano M, Griffin PT, Amorim JA, Bonkowski MS, Apostolides JK, Salfati EL, Blanchette M, Munding EM, Bhakta M *et al* (2023) Loss of epigenetic information as a cause of mammalian aging. *Cell* 186: 305-326 e327

Dear Prof. Wang

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

As you will see, all referees are very positive about the study and request only minor changes to clarify text and figures. Please add the details about the tibialis muscle injection assay to the manuscript and address the other remaining concerns/suggestions. I also agree with referee 3's suggestion to deposit the plasmids at e.g. Addgene, to provide other scientists access to these reagents. This is also in compliance with our editorial policies, as also pointed out by referee 3.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study:

- Please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- Regarding the Author Contributions, we now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section, which therefore needs to be removed from the manuscript text. You can use the free text box in our system if you wish to provide more detailed descriptions. See also guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- Please remove the list of abbreviations and make sure the define each abbreviation when it is first mentioned in the text. I am aware of the fact that referee 2 specifically asked for it, but such a list is not part of our manuscript sections.

- Please remove the figures from the manuscript .docx file.

- We can only typeset up to 5 EV figures. Maybe you can merge Figure EV6 with another figure. Otherwise, you could move some information to an Appendix (single .pdf file incl. figures and legends and a table of content with page numbers; nomenclature Appendix Fig. S#).

- Tables EV1-EV4 should be renamed as Dataset EV# and uploaded individually using the file type Data Set. Their legends need to be removed from the manuscript file and added to a separate tab of the .xls file instead.

- In Figure EV1 you re-analyse datasets published by Basisty et al 2020 and Ritchie et al 2021, if I am not mistaken. In this case, please cite the dataset in addition to the manuscript that published these data. Please see our Author guidelines, keyword 'Data citations' for more information.

- Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). This information is still missing for Prof. Liu.
Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

- Please add callouts to the panels K and L of Fig. EV3 in the text.

- Source data: Thank you for supplying all source data. I however noticed that the imaging source data for e.g., Fig. 1E-G or Fig. 2C in essence show the same image as in the figure itself and that they are supplied as part of the .pdf files. If you have the unmodified larger scans for these images, please provide these. Please also provide data for all channels individually, not only the merged images.
Preferentially, you should upload these images as individual .tif images (or another format that is re-usable)
Please see again the requirements for microscopic images:
+) Images should be provided in a common format that preserves details (e.g. .tif files). Authors are supposed to provide the original high resolution microscopic images that were used to generate the figures for the paper. If necessary, please supply a separate README file with annotation on how images were processed or arranged for the preparation of the final figure.
+) Source data files need to be submitted as zipped folders, one .zip file for each figure. Inside each folder, the files should be organized in subfolders, one subfolder for each panel. Individual files should be labeled with the assay and measured biological entities.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their

significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors have provided a detailed rebuttal following review of the manuscript and have included additional experimental evidence to support their claims. The concerns raised by this reviewer have been addressed.

Referee #2:

The authors addressed all the comments in a satisfactory manner.

Referee #3:

I would like to thank the authors for their extensive replies. The adjustments to their manuscript improved clarity and the new figures allow readers to grasp the essentials at a glance. I am now content that their manuscript is suitable for publication. Below a few additional comments which I provide for the authors so that can improve their manuscript and its impact, but do not require they address.
Plasmid sharing:
The authors addressed my comment regarding availability of their cell models:
We have written in the 'Data availability' part in the Methods -- "Other data or materials generated in this study are available from the corresponding authors upon reasonable request".
The policy of EMBO Reports states: We strongly encourage authors to deposit copies of their plasmids as DNA or bacterial stocks with repositories such as Addgene or equivalent nonprofit plasmid repository.
I would like to again encourage the authors to make their plasmids available via e.g., via Addgene after publication. While providing the guide RNA, as the authors do, of course allows other researchers to replicate their findings, sharing plasmids via public repositories with the wider scientific community is not only important for scientific progress, but it also greatly boosts the visibility of the authors' work.
Tibialis muscle injection methods:
I would suggest the authors incorporate a short version of their explanation for the tibialis muscle injection assay provided in their rebuttal letter in the manuscript (results or methods). Through the answer in their rebuttal I could understand why this assay was used, but this information is lacking in the manuscript and it would be a shame if readers do not understand and therefore discard Cheng et al. in vivo data.
Heatmap layout
The fold heatmaps the authors provided in figure 4 I/J are indeed much more useful than the previous Venn diagrams. However, to drive home the key point the authors try to make, that PDI KO reverses/ prevents expression of late-passage (senescence-associated) genes, the format could be improved. Consider that most readers will only quickly glance at figures initially, so I would suggest a format that is easier to interpret: a heatmap of (Z-scored) expression of each gene in: PDI+/+ EP, PDI +/+ LP , PDI-/- LP (proteome), PDI-/- late LP (transcriptome). I would then expect a 'blue-pink-blue-blue' row heatmap, visually indicating that indeed PDI +/- EP and PDI-/- LP are more similar than PDI+/+LP and PDI -/- LP.

Figure label subscript
(this might be an issue with the manuscript version uploaded for review only)
In some figure labels the subscript of figure labels is difficult to read due to pixelation and very small fonts. Because the subscript is used to encode essential sample information (e.g., 4I/J the +/+ and -/- behind PDI), the authors should verify that in the manuscript proof this is easily readable and if necessary, adapt the font.

Point-to-point response

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.
As you will see, all referees are very positive about the study and request only minor changes to clarify text and figures.
We thank all referees for their positive notes about this study.
1. Please add the details about the tibialis muscle injection assay to the manuscript and address the other remaining concerns/suggestions.
Response:
We have added the explanation for the tibialis muscle injection assay in the manuscript.
"The tibialis anterior muscle of mice has a microenvironment suitable for the survival of mesoderm cells and there is an accelerated attrition of senescent MSCs *in vivo* (Deng *et al*., 2019; Pan *et al*, 2016). Thus, we injected $PDI^{+/+}$ and $PDI^{-/-}$ hMSCs…" (Page 5, Line 139-141)

2. I also agree with referee 3's suggestion to deposit the plasmids at e.g. Addgene, to provide other scientists access to these reagents. This is also in compliance with our editorial policies, as also pointed out by referee 3.
Response:
We thank the editor's and referee 3's suggestion. We understand that EMBO encourage authors to deposit copies of their plasmids with repositories such as Addgene. Actually, the plasmids used in this study were modified on those from Addgene, and we have already provided the product ID and guide RNA sequence in the Methods. Thus, our CRISPR/CAS9 plasmids should be easily produced by any experienced biology laboratory. And we also promise that any materials generated in this study are available from the corresponding authors upon reasonable request. In fact, according to our experience, to deliver the plasmids directly from our lab is usually faster and cheaper than from Addgene. And we will consider to deposit the most frequently requested plasmids at Addgene in the future.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study:
3. please update the 'Conflict of interest' paragraph to our new 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest
Response:
We have made changes as requested by the editor.

4. Regarding the Author Contributions, we now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section, which therefore needs to be removed from the manuscript text. You can use the free text box in our system if you wish to provide more detailed descriptions. See also guide to authorshttps://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.
Response:

We have made changes followed the editor's request.

5. Please remove the list of abbreviations and make sure the define each abbreviation when it is first mentioned in the text. I am aware of the fact that referee 2 specifically asked for it, but such a list is not part of our manuscript sections.

Response:

We have removed the list of abbreviations from the manuscript and verified the define each abbreviation when it is first mentioned in the text.

6. Please remove the figures from the manuscript .docx file.

Response:

Done

7. We can only typeset up to 5 EV figures. Maybe you can merge Figure EV6 with another figure. Otherwise, you could move some information to an Appendix (single .pdf file incl. figures and legends and a table of content with page numbers; nomenclature Appendix Fig. S#).

Response:

We have merged Figure EV6 with Figure EV5 and changed the corresponding callouts and figure legends in the manuscript.

8. Tables EV1-EV4 should be renamed as Dataset EV# and uploaded individually using the file type Data Set. Their legends need to be removed from the manuscript file and added to a separate tab of the .xls file instead.

Response:

We have made change as required.

9. In Figure EV1 you re-analyse datasets published by Basisty et al 2020 and Ritchie et al 2021, if I am not mistaken. In this case, please cite the dataset in addition to the manuscript that published these data. Please see our Author guidelines, keyword 'Data citations' for more information.

Response:

We thank the editor for this suggestion. In fact, we did not re-analyse original datasets of cited papers. The expression change of each gene in proteome levels after multiple senescence inducers can be found in the "SASP Atlas" (www.saspatlas.com) published by Basisty et al 2020, and we have cited the website of "SASP Atlas" in the revised manuscript. The covariate associations with age in plasma proteomic can be found in Supplementary Data 2A published by Ritchie et al 2021.

10. Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>;). This information is still missing for Prof. Liu.

Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

(<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>;)

Response:

The ORCID ID of Prof. Liu has been linked to the manuscript.

11. Please add callouts to the panels K and L of Fig. EV3 in the text.

Response:

Panels K and L of Fig. EV3 have been mentioned in the text. Please have a look

"reintroduction of *PDI* into *PDI*$^{-/-}$ hMSCs increased the H_2O_2 levels in the ER (Fig 5J) and enhanced the release of H_2O_2 into the nucleus (Fig 5K and L) and cytosol (Fig EV3K and L)." (Page 10, Line 284-285).

12. Source data: Thank you for supplying all source data. I however noticed that the imaging source data for e.g., Fig. 1E-G or Fig. 2C in essence show the same image as in the figure itself and that they are supplied as part of the .pdf files. If you have the unmodified larger scans for these images, please provide these. Please also provide data for all channels individually, not only the merged images.

Preferentially, you should upload these images as individual .tif images (or another format that is re-usable)

Please see again the requirements for microscopic images:

+) Images should be provided in a common format that preserves details (e.g. .tif files). Authors are supposed to provide the original high resolution microscopic images that were used to generate the figures for the paper. If necessary, please supply a separate README file with annotation on how images were processed or arranged for the preparation of the final figure.

+) Source data files need to be submitted as zipped folders, one .zip file for each figure. Inside each folder, the files should be organized in subfolders, one subfolder for each panel. Individual files should be labeled with the assay and measured biological entities.

Response:

We thank the editor for this kind reminder. Images in Figure 1E-G, 2C, 5F were not cropped and modified, thus the source data showing the same images as in the figures.Now all original high resolution microscopic images including all channels have been provided in the corresponding zipped folders as required. And we also supply a PDF file showing how images were processed for the final figure.

13. I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

Response:

We thank the data editors' comments. We have addressed all comments and uploaded a revised file with tracked changes.

14. Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Response:

We have provided the information required along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors have provided a detailed rebuttal following review of the manuscript and have included additional experimental evidence to support their claims. The concerns raised by this reviewer have been addressed.

Response:

We thank the reviewer for his/her positive notes.

Referee #2:

The authors addressed all the comments in a satisfactory manner.

Response:

We thank the reviewer for his/her positive notes.

Referee #3:

I would like to thank the authors for their extensive replies. The adjustments to their manuscript improved clarity and the new figures allow readers to grasp the essentials at a glance. I am now content that their manuscript is suitable for publication. Below a few additional comments which I provide for the authors so that can improve their manuscript and its impact, but do not require they address.

Response:

We thank the reviewer for his/her positive notes.

Plasmid sharing:

The authors addressed my comment regarding availability of their cell models:

We have written in the 'Data availability' part in the Methods -- "Other data or materials generated in this study are available from the corresponding authors upon reasonable request".

The policy of EMBO Reports states: We strongly encourage authors to deposit copies of their plasmids as DNA or bacterial stocks with repositories such as Addgene or equivalent nonprofit

plasmid repository.

I would like to again encourage the authors to make their plasmids available via e.g., via Addgene after publication. While providing the guide RNA, as the authors do, of course allows other researchers to replicate their findings, sharing plasmids via public repositories with the wider scientific community is not only important for scientific progress, but it also greatly boosts the visibility of the authors' work.

Response:

We thank the reviewer for his/her advice. Please see our response to the editor, point 2.

Tibialis muscle injection methods:

I would suggest the authors incorporate a short version of their explanation for the tibialis muscle injection assay provided in their rebuttal letter in the manuscript (results or methods). Through the answer in their rebuttal I could understand why this assay was used, but this information is lacking in the manuscript and it would be a shame if readers do not understand and therefore discard Cheng et al. in vivo data.

Response:

We thank the reviewer for this suggestion. We have made change as the reviewer's suggestion. Please see our response to the editor, point 1.

Heatmap layout

The fold heatmaps the authors provided in figure 4 I/J are indeed much more useful than the previous Venn diagrams. However, to drive home the key point the authors try to make, that PDI KO reverses/ prevents expression of late-passage (senescence-associated) genes, the format could be improved. Consider that most readers will only quickly glance at figures initially, so I would suggest a format that is easier to interpret: a heatmap of (Z-scored) expression of each gene in: PDI+/+ EP, PDI +/+ LP , PDI-/- LP (proteome), PDI-/- late LP (transcriptome). I would then expect a 'blue-pink-blue-blue' row heatmap, visually indicating that indeed PDI +/- EP and PDI-/- LP are more similar than PDI+/+LP and PDI -/- LP.

Response:

We thank the reviewer for the insightful comment. Because the expression levels of each gene/protein from transcriptome and proteome cannot be directly compared and standardized, we choose to retain the fold heatmaps in figure 4 I/J.

Figure label subscript

(this might be an issue with the manuscript version uploaded for review only)

In some figure labels the subscript of figure labels is difficult to read due to pixelation and very small fonts. Because the subscript is used to encode essential sample information (e.g., 4I/J the +/+ and -/- behind PDI), the authors should verify that in the manuscript proof this is easily readable and if necessary, adapt the font.

Response:

We thank the reviewer for his/her advice. We have verified that the fonts in all figure labels are the same. And we have uploaded the high-resolution figures, which are easily readable.

Prof. Lei Wang
Institute of Biophysics, Chinese Academy of Sciences
National Lab of Biomacromolecules
15 Datun Road
Beijing 100101
China

Dear Prof. Wang,

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

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

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
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Corresponding Author Name: Lei Wang
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2022-56439V1

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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 manuscript.
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 replicates.
- 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 Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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?	Yes	The information has been provided in Data Availability Section.

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	The information has been provided in Materials and Methods.

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	The information has been provided in Materials and Methods

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 information has been provided in Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	The information has been provided in Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	The information has been provided in Materials and Methods

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 information has been provided in Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	The information has been provided in Materials and Methods

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 information has been provided in Acknowledgment.

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.	Not Applicable	
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.	Not Applicable	
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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
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 information has been provided in figure legends and Materials and Methods.

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 information has been provided in the related figure legends.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	The information has been provided in the related figure legends.

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	The information has been provided in Materials and Methods.
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.	Not Applicable	
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 information has been provided in Data Availability Section. The proteomic data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD037244 (Reviewer account details: Username: reviewer_pxd037244@ebi.ac.uk, Password:
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 information has been provided in the related figure legends.