EMBO reports

PPTC7 antagonizes mitophagy by promoting BNIP3 and NIX degradation via SCFFBXL4

Giang Thanh Nguyen-Dien, Brendan Townsend, Prajakta Kulkarni, Keri-Lyn Kozul, Soo Siang Ooi, Denaye Eldershaw, Saroja Weeratunga, Meihan Liu, Mathew JK Jones, S Sean Millard, Dominic CH Ng, Michael Lazarou, Michele Pagano, David Komander, Brett M Collins, Julia Pagan, Tobias Schneider, and Alexis Bonfim-Melo

Corresponding author(s): Julia Pagan (j.pagan@uq.edu.au) , Brett Collins (b.collins@imb.uq.edu.au)

Review Timeline:

Transfer Date:	21st Apr 24
Editorial Decision:	23rd Apr 24
Revision Received:	1st May 24
Editorial Decision:	14th May 24
Revision Received:	30th May 24
Accepted:	4th Jun 24

Editor: Martina Rembold

Transaction Report: A revised version of this manuscript was transferred to EMBO reports following peer review at the EMBO Journal.

(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.)

Referee #1:

The authors previously showed a role for FBXL4 in suppressing mitophagy via turnover of BNIP3 and NIX (Nguyen-Dien et al, 2023). Here, the authors explore the interaction of FBXL4 with PPTC7 phosphatase in regulating BNIP3 and NIX since like FBXL4 inactivation, loss of PPTC7 also leads to decreased mitochondria, increased mitophagy and perinatal lethality in mice. Here they show that PPTC7 is rate-limiting for the ability of FBXL4 to promote turnover of BNIP3 and NIX. They identify two forms of PPTC7, a 32 kD OMM form and a 28 kD matrix form and show that OMM PPTC7 interacts with BNIP3 and NIX, an interaction that is independent of FBXL4. Conversely, the interaction of FBXL4 with SKP1 and CUL1 is PPTC7-independent. Importantly, the authors map the sequences in BNIP3 and NIX required for PPTC7 interaction to the conserved domains carboxy terminal to the non-canonical "BH3" domain in BNIP3/NIX where a SRPE motif is particularly critical for binding to PPTC7. Also critically here the authors mutate PPTC7 in a way that inhibited its phosphatase activity without preventing interaction with BNIP3 and NIX to show that the phosphatase activity of PPTC7 is not required for the ability of FBXL4 to promote degradation of BNIP3 and NIX. This is consistent with similar work performed by Sun et al, 2024. Finally, they map the residues required for the FBXL4 interaction with PPTC7 and show tha this interaction is required for the ability of FBXL4 to promote proteasomal turnover BNIP3 and NIX.
Overall, the work is convincing and well presented with the one concern regarding novelty given the recent work from Sun et al, 2024 in Molecular Cell. The authors could have investigated further what causes the PPTC7 32 kD OMM form to accumulate which would have added a novel component here.

Major Points.
1. The authors claim that their work differs from Sun et al who propose a role for PPTC7 in recruiting CUL1 to the FBXL4 complex but they really did not test this here in their work.
2. The authors do not examine what causes OMM-PPTC7 to accumulate at the OMM which remains unanswered here or in Sun et al, 2024.

Minor Points.
1. In this paper and in the previous paper, the amino acid numbering for BNIP3 is incorrect. The SRPE motif referred to as amino acids 187 - 190 in BNIP3 should be amino acids 122 - 125. Numbering in NIX is correct. They have the numbering correct in Supplemental figure 4A, so it is unclear why they cite the wrong amino acids in the text. Sun et al, 2024 have the numbering correct for reference.

Referee #2:

In this study Nguyen-Dien and colleagues investigate regulation of BNIP3 and NIX mediated mitophagy, building from recent work by themselves that the Ub ligase complex SCF FBXL4 targets both proteins for degradation. They describe a role for the phosphatase PPTC7 in regulating this process, in essence acting as a bridging molecule between NIX, BNIP3 and FBXL4, facilitating the degradation of the NIX and BNIP3, thus counteracting mitophagy. Overall, the study is timely the data is robust and supports the authors' conclusions. I have some points for consideration during revision:

- Figure 1A - it is stated that loss of PPTC7 increases the half-life of BNIP3 and NIX however this is never quantified/demonstrated relative to WT cells (realise its challenging in the WT setting since especially BNIP3 levels are low. Would suggest the wording of this statement is changed somewhat.

- Figure 1 - Is there any effect of PPTC7 loss on BNIP3 and NIK transcript levels ? i.e. could this also contribute to high levels of NIK and/or BNIP3

- points for discussion, while the mitophagy probe does indicate increased mitophagy in the absence of PPTC7 and FKBL4, the effects are quite subtle, least throughout the level of mitochondrial protein content don't appear significantly less, some possibilities indicate additional levels of control of NIK, BNIP3 activity and/or compensatory increases in mitochondrial biogenesis.

1st Editorial Decision **23rd Apr 2024**

Dear Julia,

Thank you for the transfer of your manuscript to EMBO Reports. As my colleague at The EMBO Journal, Dr. Hartmut Vodermaier, already told you, we would like to offer rapid publication of a revised version, responding to both referees' minor points but without extension towards referee 1's major points, unless you have data at hand that might address these concerns and that you want to include. In either case, please discuss the major concerns from referee 1 in the manuscript and in a point-by-point response.

I am happy to discuss the revision further by e-mail or video call, if you wish.

Please find the general formatting guidelines below my signature. In addition, I will list here some specific points that I kindly ask you to address, as it will speed up the quality control of your revised paper and ultimately its publication.

- Please provide the Reagents and Resource table as a separate Word file (file type "Reagents and Resource table" in the manuscript tracking system). It will be typeset into the article by the production team.
- The column headers are "Reagent/Resource", "Reference or Source", "Identifier or Catalog Number".

- You need a Data availability paragraph at the end of Materials and Methods.

- You need a 'Disclosure and competing interests statement'. For more information see https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- Please provide up to 5 keywords.

- Please remove the DOIs from the reference list.

- The manuscript word file may not contain figures.

- In the figure legends, 'n' must be specified and also whether this refers to biological/independent or technical replicates. Fig 1H seems to base statistics on n = 1 with several cells quantified for each condition? If this is the case, please remove the statistical analysis.

- Please only define the p-values that are actually shown in the figure panel(s).

- If information refers to several panels in the figure (e.g., mean +/- SD, or 'n' or p-values or scale bars) then please summarize

them in a "Data Information: [....]" section at the end of the legends incl. a reference to the panels this information applies to.

- Fig S1E, H, S3C, S4B, E, Fig 5D, E either lack scale bars or they are too small/thin to be seen.
- Fig S4E lacks information on 'n'

- Supplementary figures: You could upload them as EV figures (expandable in the html version). In that case the nomenclature is Figure EV# and their legend is part of the main manuscript with the header "Expanded View Figure Legends".

- We need an Author Checklist.

- You will be contacted by our Source Data coordinator, Hannah Sonntag, listing all figure panels for which we need the minimally processed source data for. This will include all panels showing Western blots, quantification, and imaging data. You might already want to prepare these data. The structure is: One folder per figure containing subfolders for each panel. The 'figure folder' is zipped and uploaded.
An alternative is the deposition at Biostudies, in which case you insert a link that resolves to the dataset in the Data availability section.

- We also need a synopsis image (550 pixels width, 400 - 600 pixels wide, jpg or png) and a summary text (1-2 sentences), plus 3-4 bullet points.

That should be the most imporant items. General formatting guidelines are below my signature.

I look forward to receiving the revised manuscript.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO Reports

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GENERAL FORMATTING GUIDELINES:

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 (<https://www.embopress.org/page/journal/14693178/authorguide>). 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 (<https://orcid.org/>). 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>)

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: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- 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. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
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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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 and define their size in the legend, not the image.

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 <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

12) All Materials and Methods need to be described in the main text. We would encourage you to use 'Structured Methods', our new Methods format. According to this format, the Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points, to facilitate the adoption of the methodologies across labs. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in our author guidelines: < https://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation>.

An example of a Method paper with Structured Methods can be found here:
<https://www.embopress.org/doi/10.15252/msb.20178071>.

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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.

Dear Dr Rembold,

Thank you for your positive feedback on the rapid publication following the transfer of our manuscript from the EMBO Journal.

Please find below our responses to the reviewer's comments. I have attached a word document with track changes so that you can see our edits to the original manuscript.

Kind regards,

Julia

Referee #1:

The authors previously showed a role for FBXL4 in suppressing mitophagy via turnover of BNIP3 and NIX (Nguyen-Dien et al, 2023). Here, the authors explore the interaction of FBXL4 with PPTC7 phosphatase in regulating BNIP3 and NIX since like FBXL4 inactivation, loss of PPTC7 also leads to decreased mitochondria, increased mitophagy and perinatal lethality in mice. Here they show that PPTC7 is rate-limiting for the ability of FBXL4 to promote turnover of BNIP3 and NIX. They identify two forms of PPTC7, a 32 kD OMM form and a 28 kD matrix form and show that OMM PPTC7 interacts with BNIP3 and NIX, an interaction that is independent of FBXL4. Conversely, the interaction of FBXL4 with SKP1 and CUL1 is PPTC7-independent. Importantly, the authors map the sequences in BNIP3 and NIX required for PPTC7 interaction to the conserved domains carboxy terminal to the non-canonical "BH3" domain in BNIP3/NIX where a SRPE motif is particularly critical for binding to PPTC7. Also critically here the authors mutate PPTC7 in a way that inhibited its phosphatase activity without preventing interaction with BNIP3 and NIX to show that the phosphatase activity of PPTC7 is not required for the ability of FBXL4 to promote degradation of BNIP3 and NIX. This is consistent with similar work performed by Sun et al, 2024. Finally, they map the residues required for the FBXL4 interaction with PPTC7 and show tha this interaction is required for the ability of FBXL4 to promote proteasomal turnover BNIP3 and NIX.
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2. The authors do not examine what causes OMM-PPTC7 to accumulate at the OMM which remains unanswered here or in Sun et al, 2024.

We appreciate the reviewer's feedback on our manuscript. We agree with the two main points raised and believe that addressing them in future work will provide valuable insights into the function and localization of PPTC7.

Minor Points.
1. In this paper and in the previous paper, the amino acid numbering for BNIP3 is incorrect. The SRPE motif referred to as amino acids 187 - 190 in BNIP3 should be amino acids 122 - 125. Numbering in NIX is correct. They have the numbering correct in Supplemental figure 4A, so it is unclear why they cite the wrong amino acids in the text. Sun et al, 2024 have the numbering correct for reference.

Thank you for highlighting this issue, ensuring we clarify it in the current manuscript. When we initially began our research on BNIP3 several years ago, we used a DNA sequence encoding a 259 amino acid (aa) protein, as described in our previous manuscript (Nguyen-Dien). We specified this in the methods section of that manuscript (EAW49143.1 (259 aa)). We have since switched to using the 194 aa version of BNIP3 since it has replaced the 259 aa version in all databases, and our recent experiments utilize this shorter construct (note that this paper only includes experiments involving transfected NIX).

To clarify this point, we have amended the figure legend for Figure S3a to:

"Sequence alignment of BNIP3 and NIX orthologues. Functionally relevant motifs or domains are indicated. BNIP3 accession Q12983 (194 aa) replaces the previous BNIP3 accession EAW49143.1 (259 aa) characterised previously [18]."

Referee #2:

In this study Nguyen-Dien and colleagues investigate regulation of BNIP3 and NIX mediated mitophagy, building from recent work by themselves that the Ub ligase complex SCF FBXL4 targets both proteins for degradation. They describe a role for the phosphatase PPTC7 in regulating this process, in essence acting as a bridging molecule between NIX, BNIP3 and FBXL4, facilitating the degradation of the NIX and BNIP3, thus counteracting mitophagy. Overall, the study is timely the data is robust and supports the authors' conclusions. I have some points for consideration during revision:

Thank you to the reviewer for their comments.

- Figure 1A - it is stated that loss of PPTC7 increases the half-life of BNIP3 and NIX however this is never quantified/demonstrated relative to WT cells (realise its challenging in the WT setting since especially BNIP3 levels are low. Would suggest the wording of this statement is changed somewhat.

We agree that without quantification and because BNIP3 levels are so low in steady-state conditions, it is inaccurate to say that the half-life increases, therefore as suggested we have amended the text in line 97 to "levels" instead of "half-life".

- Figure 1 - Is there any effect of PPTC7 loss on BNIP3 and NIK transcript levels ? i.e. could this also contribute to high levels of NIK and/or BNIP3

We did not directly test the transcript levels of BNIP3 and NIX, however both Sun et al (Mol Cell, 84, 327, Figure 1E) and Wei et al (10.1101/2024.01.24.576953, Figure 1D) demonstrate that PPTC7 knockout does not affect BNIP3 and NIX transcript levels. We have now cited this result and refered to post-transcriptional regulation of BNIP3 and NIX in the text in line 367.

- points for discussion, while the mitophagy probe does indicate increased mitophagy in the absence of PPTC7 and FKBL4, the effects are quite subtle, least throughout the level of mitochondrial protein content don't appear significantly less, some possibilities indicate additional levels of control of NIK, BNIP3 activity and/or compensatory increases in mitochondrial biogenesis.

We have included the following text in the discussion:
Our findings show that the up-regulation of BNIP3 and NIX due to PPTC7 or FBXL4 disruption leads to mitophagy in only a subset of mitochondria. This suggests that while the increased expression of BNIP3 and NIX is necessary to trigger mitophagy, additional factors are required for its full induction, or that mitochondrial biogenesis compensates for the increased mitophagy.

Dear Julia,

Thank you for the submission of your revised manuscript to EMBO Reports.

We have completed all checks from the editorial side and I would kindly ask you to address the points below before we can proceed with the official acceptance.

Thank you very much and please do not hesitate to contact me in case you have any questions.

Kind regards,

Martina

Points to address:

1) Please make sure to cite the related manuscript from Lianjie Wei, Natalie Niemi and colleagues.

2) We noticed the following author name discrepancies:
- Giang Thanh Nguyen-Dien in the manuscript vs. Giang Nguyen-Dien in the online manuscript tracking system;
- Mathew JK Jones in the manuscript vs. Mathew Jones in the system;
- S Sean Millard in the manuscript vs. Sean Millard in the system;
- Dominic CH Ng in the manuscript vs. Dominic Ng in the system;
- Julia K Pagan in the manuscript vs. Julia Pagan in the system.
Please ensure that the information is correct and matches.

3) You state in the Author Checklist that the cell lines were tested for mycoplasma contamination. Please include this information also in the Methods section.

4) The Author Checklist will be published together with the Review Process File. Please complete the information on Corresponding Author Name, Journal Submitted to, and Manuscript Number in the file.

5) Please ensure that the funding information in the manuscript tracking system is complete, as this is the information that will be transferred to our publisher and to PubMed. In this respect we note that the Brain Foundation Research grant (2020) is missing in the onlilne system.

6) The following figure panels are never called out in the manuscript text: Figure 2BCD and Figure 6AB. Please add callouts where appropriate.

7) There are callouts to Figure 4H and EV Tables 1-3 but these are either not present in the figure (Fig 1) or missing (EV tables). This needs to be rectified.

8) You mention figures in Appendix 1A and Appendix 1B in the legend of Figure 1 but the corresponding files are missing. Please either provide these figures or correct the callouts in case you refer to an EV figure.

9) The manuscript sections should be in the following order: Title page - Abstract & Keywords - Introduction - Results - Discussion - Methods - Data Availability - Acknowledgments - Disclosure Statement & Competing Interests - References - Figure Legends - (Tables with legends) - Expanded View Figure Legends.

10) Preprint citations: Please add the prefix preprint: to the in-text citation and [PREPRINT] in the reference list.
I.e., in-text citation (preprint: NAME1 et al, YEAR) and in reference list Author NAME1, Author NAME2, (YEAR) article title. bioRxiv doi: nnn [PREPRINT]
See also https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

11) Our production/data editors have asked you to clarify several points in the figure legends (see below). Please incorporate these changes in the manuscript and return the revised file with tracked changes with your final manuscript submission.
- Please note that the legend for figure 5e is incorrectly labelled as 5i in the manuscript. This needs to be rectified.
- Please note that the legend for figure EV 4f is incorrectly labelled as EV 4h in the manuscript. This needs to be rectified.
- Please note that the exact p value is not provided in the legends of figures 4g; 5e; EV 1h; EV 4h.
- Please note that in figure 4g; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
- Please note that information related to n is missing in the legend of figure 3f.

- Although 'n' is provided, please describe the nature of entity for 'n' in the legend of figure 5e.
- Please note that the error bars are not defined in the legend of figure 3f.
- Please note that the scale bar needs to be defined for figures 3d; 5d; EV 1e, h; EV 3c; EV 4c, f.
- Please note that the red asterisk is not defined in the legend of figure 1c-d, g; 2b-e; 5c; EV 1b-c; EV 2c; EV 3d. This needs to be rectified.
- Figure 3F: please show the individual datapoints in addtion to the mean

12) It seems that the e-mail address from co-author Soo Siang Ooi (s.ooi@uq.edu.au) is not active anymore, since our e-mail bounced. Please ensure that the e-mail address is up-to-date.

13) Source data: you should be contacted by our source data coordinator Hannah Sonntag. But please have the source data ready for upload, or alternatively, upload it to BioStudies and include a link in the Data Availability section.
We will need the raw data (minimally processed) for all panels showing Western blots, immunoflourescence and quantifications (.xls files). The order is: One folder per figure with subfolders for each subpanel. This applies to all main figures, source data for EV figures is optional.

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Please provide the summary figure as a separate file in PNG or JPG format at a size of 550x300-600 pixels (width x height). 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.

15) On a different note, I would like to alert you that EMBO Press offers a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

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

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

All editorial and formatting issues were resolved by the authors.

Dr. Julia Pagan
University of Queensland
Otto Building
QLD 4067
Australia

Dear Julia,

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.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: https://www.embopress.org/page/journal/14693178/authorguide#chargesguide

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

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Reports.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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EMBO Press Author Checklist

Corresponding Author Name: Julia Pagan
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2024-59459

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

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The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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	Methods and Materials and Resource File

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	Methods and Materials and Resource File

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	Methods and Materials

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	Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Myco negative

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.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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	Acknowledgements

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.	**Yes**	

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**	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**	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figures

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 approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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?	Not Applicable	
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**.	Not Applicable	