EMBO reports

# ZEB1-mediated fibroblast polarization controls inflammation and sensitivity to immunotherapy in colorectal cancer

Constantin Menche, Harald Schuhwerk, Isabell Armstark, Pooja Gupta, Kathrin Fuchs, Ruthger van Roey, Mohammed Mosa, Anne Hartebrodt, Yussuf Hajjaj, Ana Clavel Ezquerra, Manoj Selvaraju, Carol Geppert, Stefanie Bärthel, Dieter Saur, Florian Greten, Simone Brabletz, David Blumenthal, Andreas Weigert, Thomas Brabletz, Henner Farin, and Marc Stemmler

*Corresponding author(s): Marc Stemmler (marc.stemmler@fau.de) , Thomas Brabletz (thomas.brabletz@fau.de), Henner Farin (h.farin@georg-speyer-haus.de)*

## Review Timeline:

| | |
|---|---|
| Submission Date: | 16th Apr 24 |
| Editorial Decision: | 24th Apr 24 |
| Revision Received: | 21st May 24 |
| Editorial Decision: | 3rd Jun 24 |
| Revision Received: | 10th Jun 24 |
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*Editor: Achim Breiling*

## Transaction Report: *This manuscript (previously peer reviewed and revised at a journal outside EMBO press) was transferred to EMBO reports following arbitration 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.)

Dear Dr. Stemmler,

Thank you for transferring your manuscript to EMBO reports. I now went again through the manuscript and the reports of the arbitrators from The EMBO Journal (attached again below).

Arbitrator #2 has some concerns and suggestions to improve the manuscript, I ask you to address in a final revised manuscript. Please revise your manuscript with the understanding that all concerns of the arbitrator must be addressed in the revised manuscript or in a detailed point-by-point response. As discussed in our video call, please provide further explanations and critical discussion regarding and clarify the description of the data mentioned by the arbitrator. However, we do not require that these parts of the manuscript ('claims on tumor growth, the combination with CPI and enthusiasm of therapeutic use') will be removed.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission. Please make sure that all figure panels are called out separately and sequentially in the manuscript text

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

Moreover, please consult our guidelines for figure legend preparation:
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3) a .docx formatted letter INCLUDING the arbitrators' report(s) and your detailed point-by-point responses to the 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 page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please

state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

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

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. 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.

7) 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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

9) Please note our reference format:
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10) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the author contributions section.

11) Please provide a title with not more than 100 characters (including spaces), add up to five keywords to the manuscript and order the sections like this using these names:
Title page - Abstract (175 words) - Keywords - Introduction - Results - Discussion - Methods - Data availability section (DAS) - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

12) Please add scale bars of similar style and thickness to all microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

13) Please make sure that all the funding information is also entered into the online submission system and is complete and similar to the one in the manuscript text file (in the Acknowledgements).

14) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please do not provide your final manuscript text file with an author contributions section. See also guide to authors:
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15) We would encourage you to use 'Structured Methods', our new Materials and Methods format. According to this format, the Materials and 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 (section 'Structured Methods'):

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In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

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

Kind regards,

Achim

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Achim Breiling
Senior Editor
EMBO Reports
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Arbitrator #1:

I have gone through the manuscript and rebuttal letters. My opinion is positive. The data are overall convincing and sustain the authors' conclusions. The results are novel. There have been many studies correlating CAF activity, immune evasion, and poor prognosis in CRC, but none I know shows direct genetic evidence that manipulation of CAF polarization in vivo modulates T infiltration and immunotherapy response. In addition, this study provides experimental in vivo evidence supporting the known dichotomy of CAF phenotypes; MyCAFs driven by TGF-beta (and Zeb1) and iCAFs driven by IL1. I like this paper. It is a relevant contribution.

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Arbitrator #2:

I took a close look at the paper and the reviewers comments. I fully agree with your evaluation. I believe that the existing work in breast cancer does not decrease the merit of this study. That paper (ref 25) only reports expression of Zeb1 in breast tumor CAFs. There is no functional experiment. Also, I agree that a full investigation of the mechanism underlying Zeb1 activity in CAFs seems very much outside the scope of this paper. Convincing activity should first be established and this where the challenge lies with the current study.

The issue for me is with the quality of the in vivo studies and the magnitude of the effect on tumorigenesis:
-Whether it is the DSS/AOM or the AKP model, the impact of Zeb1 deletion appears to be minor. I do not think that you can resolve this by crossing to an immune deficient background or depleting T-cells. The effect is so small and variable that this is not going to provide a clean answer.
-The impact on metastasis, if real, would be very hard to explain. It could be just decrease growth of the primary tumor but could be the inability of metastatic cells to initiate growth at the secondary side due to a change in CAF phenotype. In any case, as the effect on primary tumors is so small and variable, I do not think you can claim anything about metastasis.

-The combination experiments with Checkpoint inhibitors are very weak. If any of it was mediated by TGFb, the results would be black and white. This result has been published many time. Here is one example with 70% CR in a CRC model, MC38: (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6028240/).

In short, I would recommend to go with your second option. Minimize or remove any claims on tumor growth, the combination with CPI and enthusiasm of therapeutic use (not that anyone will get excited by Zeb1 as a therapeutic target at this time). Limit the paper to describing the change in polarization of CAFs and potential impact on immune contexture and go with EMBO Report rather than EMBO J.

Reviewers' comments:

We would like to thank both arbitrary advisors for the critical evaluation of our revised manuscript. We are grateful that both arbitrators have acknowledged our work and expressed their general support for a publication in EMBO Reports.

However, Arbitrator 2 still raises concerns on “the quality of the in vivo studies and the magnitude of the effect”. As outlined below we kindly disagree with these points and would like to stress that the unique strength of our work is that we have documented the phenotypic outcomes of CAF manipulation in three independent autochthonous/orthotopic CRC models. Our analysis shows consistent primary defects in all models (i.e. modulation of CAF subtypes, increased inflammation and immune cell infiltration) that differentially affect phenotypic outcomes in a stage and context-specific manner. We are convinced that by conducting such deep phenotypic analysis, which goes far beyond state-of-the-art, we provide a more global picture that improves our understanding on CAF biology in the dynamic tumor microenvironment. These findings have important clinical implications and will help to obtain more informative models for immune checkpoint therapy in CRC.

In a point-by-point response, we address the raised points.

Arbitrator #1:

I have gone through the manuscript and rebuttal letters. My opinion is positive. The data are overall convincing and sustain the authors' conclusions. The results are novel. There have been many studies correlating CAF activity, immune evasion, and poor prognosis in CRC, but none I know shows direct genetic evidence that manipulation of CAF polarization in vivo modulates T infiltration and immunotherapy response. In addition, this study provides experimental in vivo evidence supporting the known dichotomy of CAF phenotypes; MyCAFs driven by TGF-beta (and Zeb1) and iCAFs driven by IL1. I like this paper. It is a relevant contribution

We thank the arbitrator for his/her acknowledgement that our data are convincing and novel and that the paper is a relevant contribution.

Arbitrator #2:

I took a close look at the paper and the reviewers comments. I fully agree with your evaluation. I believe that the existing work in breast cancer does not decrease the merit of this study. That paper (ref 25) only reports expression of Zeb1 in breast tumor CAFs. There is no functional experiment. Also, I agree that a full investigation of the mechanism underlying Zeb1 activity in CAFs seems very much outside the scope of this paper. Convincing activity should first be established and this where the challenge lies with the current study.

We fully agree with the reviewer that the study by Fu et al. does not decrease the merit of our work and that precisely deciphering the molecular mechanism underlying ZEB1 activity in CAFs is outside of the scope of our study.

The issue for me is with the quality of the in vivo studies and the magnitude of the effect on tumorigenesis:

-Whether it is the DSS/AOM or the AKP model, the impact of Zeb1 deletion appears to be minor. I do not think that you can resolve this by crossing to an immune deficient background or depleting T-cells. The effect is so small and variable that this is not going to provide a clean answer.

We kindly disagree: The endpoints, as well as the associated immuno-phenotyping show consistent and significant changes in all 3 in vivo models studied. We therefore think that this criticism must have resulted from a misunderstanding. We have now more clearly explained the individual and common findings between our models (see text passages labelled in red and graphical abstract). Notably, we found increased immune cell infiltration in FibΔZeb1 mice in all 3 models. The observed differences in specific immune cell subsets are likely a result of the fundamentally distinct etiologies of the studied models. In the inflammation-driven tumor model (AOM/DSS) we observed increased adenoma formation (size and number). This is compatible with our hypothesis that the increased inflammatory response in AOM/DSS tumorigenesis in combination with the altered inflammatory CAF subtype upon Zeb1 depletion increases tumor initiation and growth. This idea is further supported by the finding that the primary tumor growth was unaffected in both sporadic models (orthotopic transplantation and AOM/p53). In contrast, the consequences of ZEB1 dependent CAF modulation in advanced stages could only be addressed in the sporadic models because the AOM/DSS tumors remain benign. Here, we could observe that *Zeb1* deletion is associated with decreased local invasion (AOM/p53) and distant metastasis (orthotopic transplantation), indicating that the CAFs modulation can counteract tumor progression. Consistent with the common increase of immune cell infiltration we found that immune checkpoint therapy resulted in reduced tumor burden in both sporadic and inflammation induced tumors. Together, our results highlight how ZEB1 in CAFs drives tumor-TME crosstalk in a context and stage-specific manner, which commonly results in increased immune cell infiltration. Furthermore, we agree that experiments in an immunodeficient background or T-cell depletion would not add substantially new mechanistic insights.

-The impact on metastasis, if real, would be very hard to explain. It could be just decrease growth of the primary tumor but could be the inability of metastatic cells to initiate growth at the secondary side due to a change in CAF phenotype. In any case, as the effect on primary tumors is so small and variable, I do not think you can claim anything about metastasis.

We agree that the exact mechanism how ZEB1 affects tumor progression by modulating CAF composition and immune cell infiltration remains open. However, we are puzzled that the reviewer questions the general relevance of our observations. Notably, we show significant and profound reduction of metastasis incidence and number in spontaneous transplantation models that provide a high physiologic relevance (Fig. 1K, L) Because, we did not observe any parallel reduction of primary tumor growth this points towards a specific anti-metastatic effect. While our data does not exclude an additional role of ZEB1 in CAFs at the secondary site, we show significantly increased T and B-cell infiltration in the primary tumors arguing for increased anti-tumor immunity (Fig. 4B) that was independently observed also in the AOM/p53 model (Fig. EV4). In summary, we are convinced that our accumulated data provide consistent evidence about this key aspect of our analysis indicating that ZEB1 orchestrates CAF diversification and formatting of the TME, which has major impact on tumor biology and immunity.

-The combination experiments with Checkpoint inhibitors are very weak. If any of it was mediated by TGFb, the results would be black and white. This result has been published many time. Here is one example with 70% CR in a CRC model, MC38: (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6028240/).

Given the clear and consistent changes described in Fig. 5 and Fig. EV5 we are surprised that the arbitrator considers our immune checkpoint blockade experiments as “weak”. We think that referring to a study in which 2D cell lines have been subcutaneously injected for

ICB treatment is rather inadequately challenging our results by ignoring that we exclusively used autochthonous and orthotopic transplantation models with high clinical relevance. We also need to stress that MC38 cells are known to be responsive to ICB (hence a 70% CR), which holds true only for a minority of patient's CRC tumors. The importance of our findings is that in our models neither AOM/DSS or sporadic tumors were responsive to ICB, but depleting *Zeb1* from CAFs and the subsequent TME remodeling rendered these tumors responsive to ICB. In the orthotopic model the effect on tumor onset is maybe not substantial as we focused on a clinically relevant setting and started treatment once tumors are about to become apparent (14 d after inoculation). In contrast, the effect is remarkably solid as with just three times of anti-PD-L1 treatment within 3 weeks the tumor volume was reduced to 1/2 and the effectiveness of ICB was confirmed by expected changes on immune cell markers. Similarly, in AOM/DSS tumors we do not observe response to ICB in Fibctrl mice, but a strong reduction in tumor growth, with some tumors even reducing their size, whereas others did not respond so well. This observation goes along with the relatively high variability in tumor size observed in this model upon *Zeb1* depletion.

We agree that among the many functions of TGFβ in CRC, high TGFβ signals are correlated with poor prognosis and refractoriness to ICB. We have shown that *Zeb1*-deficent CAFs are less responsive to TGFβ and more prone to adopt and maintain an altered iCAF phenotype (Fig. 4). However, we did not analyze the TGFβ abundance and modulation of TGFβ would certainly also have consequences on other cell types in the TME. As *Zeb1* depletion in CAFs shifts the diversity of CAFs and alters their function to attract immune cells, which goes beyond the role of TGFβ alone, we exclude a major TGFβ-driven effect.

In short, I would recommend to go with your second option. Minimize or remove any claims on tumor growth, the combination with CPI and enthusiasm of therapeutic use (not that anyone will get excited by Zeb1 as a therapeutic target at this time). Limit the paper to describing the change in polarization of CAFs and potential impact on immune contexture and go with EMBO Report rather than EMBO J.

We welcome that the arbitrator recommends our study for publication in EMBO Reports. We thoroughly went through the text and found a few instances where further toning down of conclusions deemed appropriate (labeled in red). We also explicitly addressed the "limitations of the study" in the last section of the discussion. Moreover, we now provide a graphical summary, in addition to minor text modifications to better explain unique features and the consistent findings in the different models.

Dear Dr. Stemmler,

Thank you for the submission of your further revised manuscript to our editorial offices. I now went through this and your p-b-p-response and consider the points of advisor #2 as adequately addressed.

Before proceeding with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please provide a final title with not more than 100 characters (including spaces).

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions and do NOT provide your final manuscript text file with an author contributions section. See also our guide to authors:
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- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures). Please also check that the exact p-values are indicated in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams. Presently, diagrams seem to miss the 'n.s.'. Please check.

Moreover:
- Please note that the exact p values are not provided in the legends of figures 1d, f-g, k-l; 2d; 3b, d-e, g-h; 4a-c, e-g, i; 5a-b, d; EV 1a; EV 3d; EV 4b-c, e; EV 5d-f.
- Please indicate the statistical test used for data analysis in the legends of figures 2d, i-j; EV 2c-d.
- Please note that in figures 4f; EV 4e; 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 5d.
- Although 'n' is provided, please describe the nature of entity for 'n' in the legends of figures 3b; 4c; EV 1h, k; EV 3d.
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- Please add to each legend (main, EV and Appendix figures, where applicable) a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales. See:

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- Please add specific URLs that lead to the datasets GSE253368, GSE253639 and GSE253546 to the data availability statement, and make sure these datasets are public latest upon online publication of the manuscript.

- Co-corresponding author Thomas Brabletz is an EMBO reports board member. Please add the following sentence to the 'Disclosure and competing interests statement': "Thomas Brabletz is a member of the Advisory Editorial Board of EMBO reports. This has no bearing on the editorial consideration of this article for publication."

- There is a Fig. 2M called out in the manuscript text (page 7), but there seems to be no such panel. Please check.

- Please change the callouts for the Appendix Tables to 'Appendix Table Sx' (the "S" is presently missing).

- Thanks for providing the numerical source data (SD). You should have been contacted by our source data coordinator with information on which figure panels we would need source data for. I attach again the source data checklist. Please make sure that all the requested source data is provided. Please upload all source data for one figure ZIPed together as one folder. Please also upload the filled in source data checklist with your final revised files. Additional SD for EV and Appendix figures can be zipped up together in one folder.

- The schematic summary figure you have provided has not the right format. Scaling it down would render the text hardly readable. Please provide a final schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels.

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

regarding the revision.

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

Best,

Achim Breiling
Senior Editor
EMBO Reports

All editorial and formatting issues were resolved by the authors.

Dr. Marc Stemmler
Friederich-Alexander University of Erlangen-Nürnberg
Experimental Medicine 1
Glückstr. 6
Erlangen 91054
Germany

Dear Dr. Stemmler,

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.

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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.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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|---|
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| Journal Submitted to: EMBO Reports |
| Manuscript Number: EMBOR-2024-59433-T |

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The EMBO Journal - Author Guidelines
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Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

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

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

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

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

| Experimental animals | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Laboratory animals or Model organisms:** Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID. | Yes | 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**. | 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 section |

## Design

| Study protocol | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI. | 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. | Yes | Material and Methods |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Yes | Material and Methods |
| Include a statement about **blinding** even if no blinding was done. | Yes | Material and Methods |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Yes | Material and Methods |
| 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 | Material 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 | Figure Legends, Expanded view Figure Legends |
| In the figure legends: define whether data describe **technical or biological replicates**. | Yes | Figure Legends, Expanded view 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 approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations. | Yes | Material 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 | Material and Methods |
| 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? | Yes | Material and Methods |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Not Applicable | |