EMBO reports

Sirt6 ablation in the liver causes fatty liver that increases cancer risky by upregulating Serpina12

Licen Li, Jianming Zeng, Xin Zhang, Yangyang Feng, Josh Lei, Xiaoling Xu, Qiang Chen, and Chu-Xia Deng

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

Dear Prof. Deng,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

The referees express interest in the proposed role of Sirt6-Serpina12 link in HCC progression. However, they also raise significant concerns that need to be addressed to consider publication here.

While we agree with referee #2 that elucidating the mechanism by which Serpina12 regulates expression of genes related to lipogenesis would significantly strengthen the manuscript (point 3), not being able to do so will not preclude from publication here.

Given these positive recommendations, we would like to invite you to submit a revised manuscript. Please revise your manuscript with the understanding that the referee concerns (as 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 major experimental 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. Please discuss the revision progress ahead of this time with me if you require more time to complete the revisions, or if you have questions or comments regarding the revision (also by video chat).

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:
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2. Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases.

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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). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

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https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
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9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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- 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 Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_revise_and_review.txt line 56.' 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.

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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.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In this study, Deng and colleagues investigated the role of hepatic SIRT6 in regulation of HCC in vitro and in vivo.

Overall, this is a thorough and comprehensive study, containing well-designed in vivo phenotypic characterizations, solid in vitro mechanistic analyses and validations. The data is compelling, and all results are convincing. I only have a few suggestions/questions/comments for the improvement of this study:

1. The authors labeled their control mice as "WT" mice. But they are in fact Sirt6 flox/flox mice based on my understanding. Suggest using the term of "Flox" instead of "WT" for these control mice.
2. One interesting observation is that SIRT6 LKO mice have reduced immune cells in the liver in both regular condition (Figure S1D) and after breeding into the ob/ob background (Figure S7I). The authors showed that there were reduced abundance of CD4+ and CD8+ T cells in Figure S7I. Have the authors checked Kupffer cells, the liver resident macrophages, in SIRT6 LKO mice under both conditions? Also, can the authors discuss the possible implications of this reduced immunity in promoting hepatic tumorigenesis in SIRT6 LKO mice?
3. Figure 3, very nice results! Two questions: does C/EBPa directly interact with SIRT6 so that SIRT6 can be specifically target to the promoter of Serpina12 gene? Could the authors confirm that the binding of C/EBPa is important for SIRT6 to suppress the expression of Serpina12 by generating mutant luciferase reporters that lack functional C/EBPa binding site (site 1 in Figure 3C), then show that SIRT6 repress the WT but not mutant luciferase reporter in hepatocytes?
4. Figure 4, does the SIRT6 level change (reduce) during high glucose or oleic acid induced lipogenesis in hepatocytes?
5. Figure 5, nice data. Have the authors analyzed the expression of SIRT6 in NASH? How about the correlations between SIRT6 and SERPINA12 as well as between SIRT6 and patient survival in HCC patients?

Referee #2:

In this study, Li et al demonstrated that depletion of Sirt6 in mice liver resulted in alteration of H3K9 and H3K56 acetylation and gene expression profile. By comparison of the RNA-seq data and ChIP-seq data from wild type and Sirt6-LKO mice live, Serpina12 was identified as the key gene downstream of Sirt6. Ablation of Sirt6 increased the expression level of Serpina12 by enhancement of the level of H3K9ac and H3K56ac and the binding of transcription factor CEBPα. Increased expression of Serpina12 in hepatocytes enhanced insulin signaling and lipid accumulation. In addition, Sirt6 was shown to be a tumor suppressor in liver. Although the work might be interested, several concerns are raised.
1.The authors demonstrated the depletion of Sirt6 in hepatocyte resulted in high level of SERPINA12 to enhance insulin signaling and promote lipid accumulation. Knock out of Sirt6 was shown to cause a lipid rich environment to accelerate hepatocellular carcinoma (HCC). Since insulin signaling was also reported to play important role in tumorigenesis and progression. The authors should evaluate the effect of insulin signaling in HCC.
2.SERPINA12 was reported to be an extracellular serine protease inhibitor which inhibited the activity of serine protease like KLK7 to increase the concentration of insulin in serum and enhance insulin signaling. In this study, Serpina12 was shown to promote expression of genes related to glucose and lipid metabolism. Therefore, the author should demonstrate that SERPINA12 can be transported into nucleus.
3.An interesting question should be addressed is the mechanism of SERPINA12 be recruited to chromatin. Either the DNA motif or the protein factors targeting SERPINA12 to chromatin should be identified.

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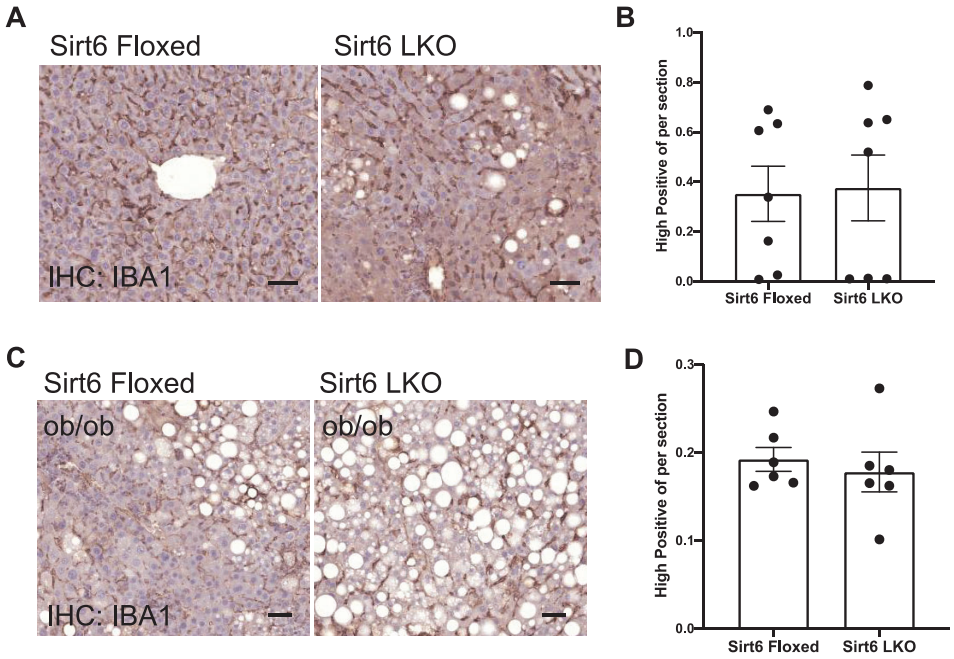
1. The authors labeled their control mice as "WT" mice. But they are in fact Sirt6 flox/flox mice based on my understanding. Suggest using the term of "Flox" instead of "WT" for these control mice.

Thank you for your suggestion. We have changed the term "WT" to "Floxed" accordingly in the revised manuscript.

2. One interesting observation is that SIRT6 LKO mice have reduced immune cells in the liver in both regular condition (Figure S1D) and after breeding into the ob/ob background (Figure S7I). The authors showed that there were reduced abundance of CD4+ and CD8+ T cells in Figure S7I. Have the authors checked Kupffer cells, the liver resident macrophages, in SIRT6 LKO mice under both conditions? Also, can the authors discuss the possible implications of this reduced immunity in promoting hepatic tumorigenesis in SIRT6 LKO mice?

For detecting Kupffer cells, we did IHC with an antibody to IBA1, which is Kupffer cells marker in the liver in Sirt6 LKO mice in both conditions. There were no obvious changes in both conditions (R-Figure 1A and 1B).



R-Figure 1 (A) IHC staining of IBA1 in Sirt6 Floxed and Sirt6 LKO mice. (B) IBA1 IHC staining quantification. (C) IHC staining of IBA1 in Sirt6 Floxed and Sirt6 LKO mice under the condition of obesity. (D) IBA1 IHC staining quantification.

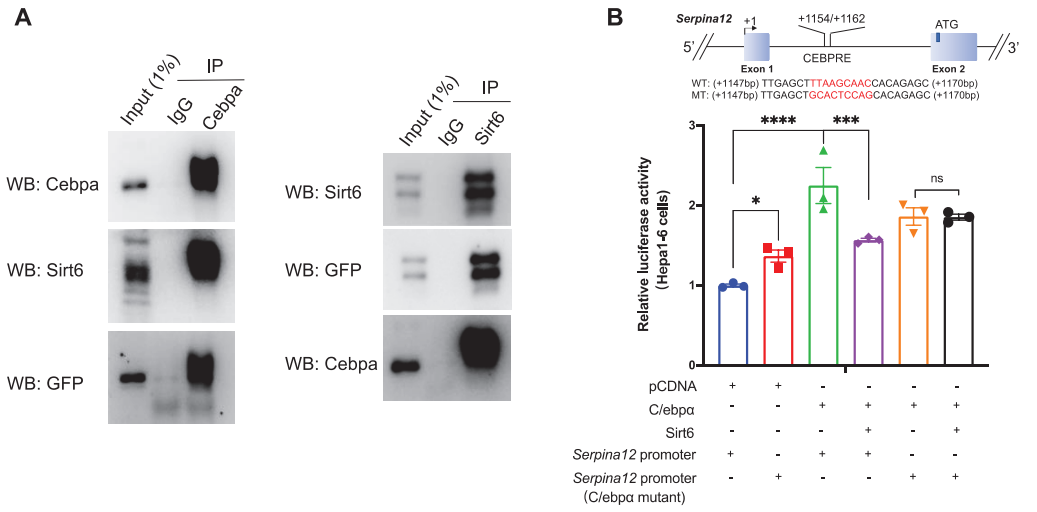
We observed that the immune response related pathway was decreased in Sirt6 LKO mice using RNA-seq

analysis. These pathways were downregulated, suggesting the weaker activity of the immune cells in the Sirt6 LKO mice liver. Although we found that the number of Kupffer cells has no obvious change in the liver, T cells could also affect immune processes. In Sirt6 LKO mice with obesity background, we have found reduced CD4+ and CD8+ T cells in the liver. The reduced immunity could lead to the failure to eliminate cancer cells in the liver, thus promoting the tumor formation. However, the relationship between the immune system and cancer is complex, and enhancing the immune system alone may not be effective to treat liver cancer in clinical condition. Further study is needed to illustrate the effect in this case. The possible implications were discussed in the revised manuscript. We have indicated in the Discussion that the reduced CD4+ and CD8+ T cells in the liver could lead to the failure to eliminate cancer cells in the liver, thus promoting the tumor formation (page 28, line 1-2, line 11-19 and page 29 line 1-6).

3. Figure 3, very nice results! Two questions: does C/EBPa directly interact with SIRT6 so that SIRT6 can be specifically target to the promoter of Serpina12 gene? Could the authors confirm that the binding of C/EBPa is important for SIRT6 to suppress the expression of Serpina12 by generating mutant luciferase reporters that lack functional C/EBPa binding site (site 1 in Figure 3C), then show that SIRT6 repress the WT but not mutant luciferase reporter in hepatocytes?

To determine whether C/EBPa could directly interact with SIRT6, we induced Sirt6-GFP and C/ebpα expression 48h in 293T cells and performed a co-immunoprecipitation assay with antibodies against Sirt6 and C/ebpα. The results showed that CEBPA could directly interact with SIRT6 (R-Figure 2A).

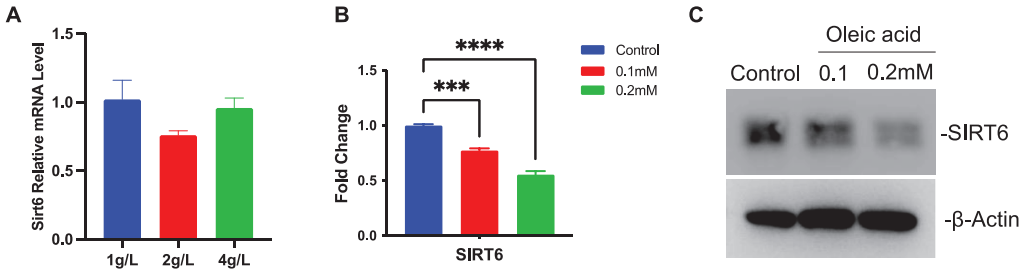
To address the second question, we generated a mutant luciferase reporter that lacks functional C/EBPa binding site. The data indicated that Sirt6 could suppress the increased expression of WT Serpina12-luc activity induced by C/EBPa. In contrast, such suppression was largely abolished after the mutation of the binding site of C/EBPa (R-Figure 2B). This observation indicates that the binding of C/EBPa is important for SIRT6 to suppress the expression of Serpina12. This data was placed in the Figure Extended View 3D and 3E and the corresponding text (page 13 and line 6-11).



R-Figure 2 (A) IP experiments on lysates from 293T/17 cells detected by antibodies of Sirt6 and C/ebpα. Sirt6-GFP and C/ebpα expression was induced 48 h prior to IP. (B) Ectopic expression of C/ebpα increased Serpina12 promoter activity, whereas mutation of C/ebpα-binding sites reduced the activity. Ectopic expression of Sirt6 represses the Serpina12 promoter activity but not the mutant C/ebpα-binding sites promoter activity.

4. Figure 4, does the SIRT6 level change (reduce) during high glucose or oleic acid induced lipogenesis in hepatocytes?

Thank you for your comment. We have observed that Sirt6 level has no significant change in 1g/L (low glucose) compared to 4g/L (high glucose) both at RNA (R-Figure 3A) and protein levels (manuscript Figure 4D). However, the SIRT6 level has significantly decreased after oleic acid treatment in HepG2 cells (R-Figure 3B and 3C), suggesting that the expression change of Sirt6 might be more sensitive to condition under lipogenesis.

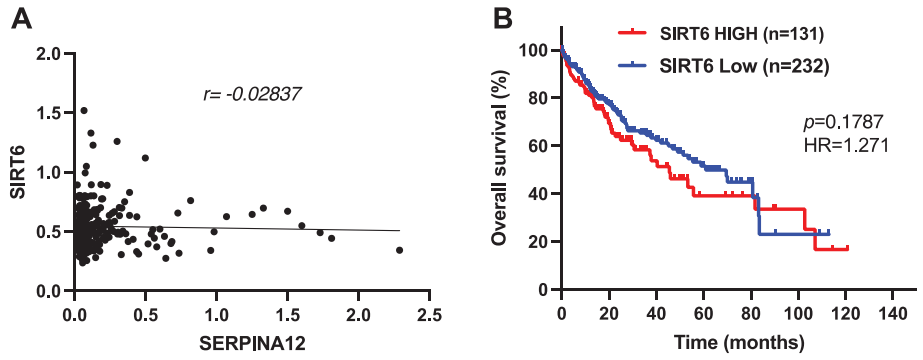


R-Figure 3 (A) qPCR analysis of Sirt6 mRNA levels in different glucose concentration. (B) qPCR analysis of Sirt6 mRNA levels in different oleic acid concentration treatment. (C) Western blot analysis of Sirt6 mRNA levels in different oleic acid concentration treatment.

5. Figure 5, nice data. Have the authors analyzed the expression of SIRT6 in NASH? How about the correlations between SIRT6 and SERPINA12 as well as between SIRT6 and patient survival in HCC patients?

Thank you for the suggestion. We checked the expression of SIRT6 in NASH patients in a GEO database and found that it was at a low expression level compared to other genes (revised manuscript Figure 5D).

We found a negative correlation between Sirt6 and Serpina12 in the patients with HCC in a GSE25097 database (R-Figure 4A). To understand the role of SIRT6 for patient survival, we analyzed the TCGA database in HCC patients. We defined SIRT6 high or SIRT6 low expression based on the mean value in these HCC patients and the data indicated that overall survival has no significant difference between SIRT6 high and SIRT6 low expression (R-Figure 4B).



R-Figure 4 (A) Pearson correlation analysis of SIRT6 and SERPINA12 mRNA expression in HCC (GSE25097). (B) Kaplan-Meier curves for overall survival of HCC patients with high and low SIRT6.

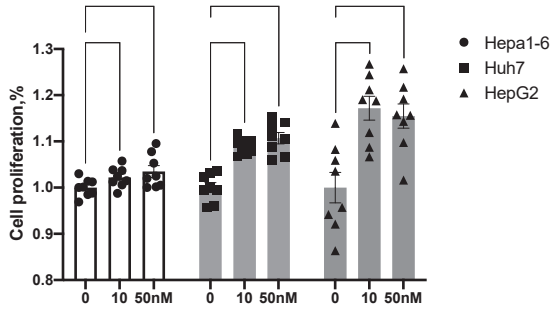
Referee #2:

In this study, Li et al demonstrated that depletion of Sirt6 in mice liver resulted in alteration of H3K9 and H3K56

acetylation and gene expression profile. By comparison of the RNA-seq data and ChIP-seq data from wild type and Sirt6-LKO mice live, Serpina12 was identified as the key gene downstream of Sirt6. Ablation of Sirt6 increased the expression level of Serpina12 by enhancement of the level of H3K9ac and H3K56ac and the binding of transcription factor CEBPα. Increased expression of Serpina12 in hepatocytes enhanced insulin signaling and lipid accumulation. In addition, Sirt6 was shown to be a tumor suppressor in liver. Although the work might be interested, several concerns are raised.

1. The authors demonstrated the depletion of Sirt6 in hepatocyte resulted in high level of SERPINA12 to enhance insulin signaling and promote lipid accumulation. Knock out of Sirt6 was shown to cause a lipid rich environment to accelerate hepatocellular carcinoma (HCC). Since insulin signaling was also reported to play important role in tumorigenesis and progression. The authors should evaluate the effect of insulin signaling in HCC.

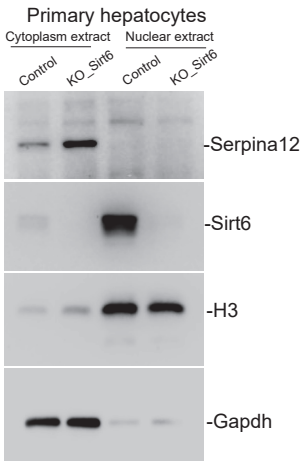
Thank you for the suggestion. Insulin-dependent signaling pathways are often overactivated in human HCC due to the overexpression of signaling components and loss of negative regulators[1, 2]. As a result, hyperinsulinemia can directly promote cancer cell metabolism, proliferation, and survival, potentially contributing to HCC development and progression[3]. We have tested the effect of insulin signaling in HCC and the data indicated that insulin signaling plays a crucial role in the development of hepatocellular carcinoma (HCC) by promoting cell proliferation (R-Figure 5). The data is placed in the revised manuscript Appendix Figure S3, and the corresponding text (page 26, line 6-12).



R-Figure 5 Cell proliferation rate with different dose of insulin treatment.

2. SERPINA12 was reported to be an extracellular serine protease inhibitor which inhibited the activity of serine protease like KLK7 to increase the concentration of insulin in serum and enhance insulin signaling. In this study, Serpina12 was shown to promote expression of genes related to glucose and lipid metabolism. Therefore, the author should demonstrate that SERPINA12 can be transported into nucleus.

Thank you for the suggestion. To understand the location of Serpina12, we isolate cytoplasm extract and nuclear extract in primary hepatocytes. The data that Serpina12 was in cytoplasm in the cells, but not in the nucleus (R-Figure 6). We have also demonstrated that knockdown Sirt6 increased Serpina12 expression in the cytoplasm, which may trigger the lipid accumulated in the cells. Thus, effect of Serpina12 on genes related to glucose and lipid metabolism might be caused other mechanism rather than directly regulates their expression.



R-Figure 6 Western blot analysis of Serpina12 in cytoplasm extract or nuclear extract with or without knockout Sirt6 in primary hepatocytes.

3. An interesting question should be addressed is the mechanism of SERPINA12 be recruited to chromatin. Either the DNA motif or the protein factors targeting SERPINA12 to chromatin should be identified.

In our study, we found that Sirt6 affects the Histone 3 lysine 9 acetylation, and they have increased binding peaks in the Serpina12 locus. As Serpina12 is only in the cytoplasm, but not in the nucleus (R-Figure 6), it is unlikely that Serpina12 could be recruited to chromatin.

[1] Alqahtani, A., Z. Khan, A. Alloghbi, et al., Hepatocellular Carcinoma: Molecular Mechanisms and Targeted Therapies[J]. Medicina (Kaunas), 2019. **55**(9).

[2] Enguita-German, M., P. Fortes, Targeting the insulin-like growth factor pathway in hepatocellular carcinoma[J]. World J Hepatol, 2014. **6**(10): 716-37.

[3] Chettouh, H., M. Lequoy, L. Fartoux, et al., Hyperinsulinaemia and insulin signalling in the pathogenesis and the clinical course of hepatocellular carcinoma[J]. Liver Int, 2015. **35**(10): 2203-17.

1st Revision - Editorial Decision **7th Dec 2023**

Dear Prof. Deng,

Thank you for submitting your revised manuscript. It has now been seen by both of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. However, I need you to address the points below before I can accept the manuscript.

• Please remove the figures from the main manuscript file and reorder the sections as follows: Title page - Abstract & Keywords - Introduction - Results - Discussion - Materials & Methods - Data Availability - Acknowledgments - Disclosure Statement & Competing Interests - References - Figure Legends - Tables with legends - Expanded View Figure Legends.
• Please remove the Author Contributions section from the manuscript.
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• Please add more details about the experiments performed, statistical tests applied and the meaning of *, *** and **** to the legend of Appendix Figure S3.
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Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The revision addressed my major concerns.

Referee #2:

The authors addressed my previous concerns. I have no question now.

All editorial and formatting issues were resolved by the authors.

Dear Prof. Deng,

Thank you for submitting your revised manuscript.My apologies for the unusual delay in getting back to you. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Scientific Editor
EMBO Reports

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	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 χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Materials

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

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

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	

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	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	
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	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	

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	

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.	Yes	

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	
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	
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	

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

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	
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	
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	